[illegible]
5506
Industrial

T2 18

# INDUSTRIAL MICROBIOLOGY

**SAMUEL CATE PRESCOTT, Sc.D.**
*Professor of Industrial Biology (Emeritus), Former*
*Head of the Department of Biology and Public*
*Health and Dean of the School of Science*
*Massachusetts Institute of Technology*

**CECIL GORDON DUNN, Ph.D.**
*Associate Professor of Industrial Microbiology*
*in the Department of Food Technology*
*Massachusetts Institute of Technology*

THIRD EDITION
*Revised by*
*Cecil Gordon Dunn*

McGRAW-HILL BOOK COMPANY, INC.
NEW YORK TORONTO LONDON
1959

J9
5906
Gv-9x
N59

INDUSTRIAL MICROBIOLOGY

Copyright © 1959 by the McGraw-Hill Book Company, Inc.

Copyright, 1940, 1949, by the McGraw-Hill Book Company, Inc. Printe in the United States of America. All rights reserved. This book, or part thereof, may not be reproduced in any form without permission o the publishers. *Library of Congress Catalog Card Number* 58-1000

II

50748

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE * MYSORE. *
LIBRARY

CFTRI-MYSORE
5906
Industrial micro.

# PREFACE

It is the purpose of this volume to outline, in a concise but comprehen-ive manner, the fundamentals of industrial microbiology and to present escriptions of the more important processes within the field.

No single text of moderate size can present a complete and detailed reatment of every aspect of the subject. In order to provide a guide for dditional reading, lists of selected references are given at the ends of ost of the chapters.

In the present revision, new chapters have been added relating to the ransformation of steroids, the production of amino acids, dextrans, gib-erellic acid and the gibberellins, mushroom mycelium, vitamins, and iochemical engineering aspects of industrial microbiology. Large por-ons of a number of chapters have been rewritten and expanded. New ables and figures have been added. Certain less pertinent material has een deleted.

In the preparation of this book, the authors have received help in a umber of forms: permission to reproduce figures and tables, reviewing f chapters or sections, supplying of reprints, reading of the manuscript nd proofs, encouragement, and aid in the typing.

Thanks and appreciation are extended to the publishers and editors of e journals, to the authors, and to others who have so kindly granted ermission for the reproduction of figures and/or tables. In this connec-ion, the authors wish to mention especially the kindness of the following: he American Chemical Society, publishers of *Industrial and Engineering hemistry* (Dr. Will H. Shearon, Jr., editor), *Journal of Agricultural and ood Chemistry*, and *Journal of the American Chemical Society;* Leonard ill, Ltd., publishers of *Food Manufacture*; North Holland Publishing o. (Dr. M. D. Frank), publishers of *The Yeasts,* by Lodder and Kreger an-Rij; The Williams & Wilkins Company (Dr. E. F. Williams), pub-shers of *Applied Microbiology, Bacteriological Reviews,* and *Journal of acteriology;* and the publishers of *American Brewer* (Rodney Leeds), *rchives of Biochemistry and Biophysics, Biochemical Journal* (Dr. F. lark), *Canadian Journal of Botany, Canadian Journal of Microbiology, ereal Chemistry* (Dr. R. A. Bottomly), *Enzymologia* (Dr. W. Junk), *od Engineering, Food Technology, Journal of Dairy Science* (Dr. E. F. elson), *Journal of General Microbiology* (Dr. A. F. B. Standfast), *Jour-l of the Science of Food and Agriculture, Laboratory Practice, Mycolo-*, Swets and Zeitlinger (publishers of *Antonie van Leeuwenhoek*); the *llerstein Laboratories Communications* (Dr. Philip Gray); Anheuser-

Busch, Inc.; Carling Brewing Company; Chas. Pfizer and
Commercial Solvents Corp.; Lederle Laboratories Division,
Cyanamid Company; Merck and Company; the U.S. Industri
cals Co., division of National Distillers and Chemical Corp.;
Upjohn Company. The kindnesses of the following individuals
associates are greatly appreciated: C. G. Anderson, H. R. V.
T. Asai, T. H. Backus, F. W. Beech, S. C. Beesch, S. S. Bl
Bu'Lock, L. V. Burton, L. Chaiet, C. F. Cori, W. V. Cruess, A. L
E. L. Dulaney, E. O. Ericsson, J. L. Etchells, F. W. Fabian, J
C. N. Frey, H. Frings, G. A. Geffreys, P. P. Gray, A. Guilliermo
Herrick, H. E. Howe, O. Hromatka, H. Humfeld, G. C. Inskee
Jackson, M. J. Johnson, I. D. Jones, H. Koethe, P. Kolachov, A.
ver, H. S. Knight, N. V. W. Kreger van-Rij, G. A. Ledingha
Lemieux, C. P. Lentz, J. C. Lewis, C. C. Lindegren, G. O. Lin
Lockwood, J. Lodder, O. E. May, E. J. McCann, O. Meyerhof, A. J
E. M. Mrak, S. Otsuka, G. W. Packowski, D. H. Peterson, W.
son, V. F. Pfeifer, H. J. Phaff, H. Raistrick, K. Raper, W. P. R
A. Robertson, M. Rogosa, E. A. Rossi, J. F. Saeman, R. Schn
Schwarz, F. R. Senti, S. Shibater, G. M. Shull, G. B. Sippel, L.
J. F. T. Spencer, A. R. Stanley, R. Steel, M. Stephenson, T. R.
F. R. Swift, T. H. M. Taylor, W. C. Tobie, L. A. Underkofle
Waksman, T. K. Walker, C. H. Werkman, J. White, T. Wikén, Ö
and K. Yamada. Special thanks are extended to Mrs. Cecil G. D
her help in reading the manuscript and proofs; and to Mrs. E
Dunn Hills, for her encouragement. The help of Virginia Bisho
Frey, Janet Hartwell, and Virginia Pochetti in typing parts of th
script is much appreciated.

Grateful acknowledgment is made to Professor Bernard E.
Head of the Department of Food Technology of the Massachuset
tute of Technology, who made it possible for Cecil G. Dunn to sp
of his work time on the revision of the book.

*Cecil G.*

# CONTENTS

*Preface* v

Chapter 1. Introduction 1
2. The Yeasts 10
3. The Propagation of Yeast 60
4. The Production of Industrial Alcohol by Fermentation 102
5. Facts and Theories Concerning the Mechanism of Ethyl Alcohol Fermentation 128
6. Brewing 148
7. Wine 174
8. The Distilling Industries 195
9. The Glycerol Fermentation 208
10. Vitamin Production by Yeasts and Yeast-like Microorganisms 218
11. Yeast Enzymes and Minor Products 236
12. The Bacteria 247
13. The Acetone-Butanol Fermentation 250
14. The Butanol-Isopropanol Fermentation 285
15. The Acetone-Ethanol Fermentation 295
16. The Lactic Acid Bacteria 299
17. The Production of Lactic Acid by Fermentation 304
18. Sauerkraut 332
19. Pickles 341
20. Dextrans 370
21. Cheese 393
22. The Production and Properties of 2, 3-Butanediol 399
23. The Acetic Acid Bacteria and Some of Their Biochemical Activities 428
24. The Propionic Acid Fermentation 474
25. The Production of Vitamin $B_{12}$ 482
26. Some Minor Bacterial Activities with Industrial Implications 497
(enzym)
27. The Molds 515
28. The Citric Acid Fermentation 533
29. The Gluconic Acid Fermentation 578
30. The Itaconic Acid Fermentation 598
31. The Kojic Acid Fermentation 609
32. Gibberellic Acid and the Gibberellins 619

Chapter 33. The Fumaric Acid Fermentation
34. The Production of Lactic Acid by Molds
35. The Gallic Acid Fermentation
36. Ustilagic Acid
37. Some Other Acids Produced by Molds
38. Mannitol Production by Molds
39. Production of Mushroom Mycelium by the Submerged-culture Process
40. The Production of Fat by Molds
41. Mold Enzyme Preparations: Uses and Products
42. Some Minor Chemical Activities of the Lower Fungi
43. The Production of Amino Acids by Microorganisms eg-Gl
44. The Microbiological Transformation of Steroids
45. Antibiotics
46. Saccharifying Agents: Methods of Production and Uses
47. Some Biochemical Engineering Aspects of Fermentation

*Index*

CHAPTER 1

# INTRODUCTION

Industrial microbiology is that portion of microbiological science which deals with the possible utilization of microorganisms in industrial processes, or in processes in which their activities may become of industrial or technical significance. Obviously the term "microbiology" in its broadest sense comprises that division of biological science which treats of the extremely small organisms of both plant and animal natures that Sëdillot in 1878 grouped together under the inclusive term "microbes." Although this word is now often used synonymously with bacteria, it is not so limited, since within its field may actually be included all types of very small living things, such as the protozoa, spirochetes, and minute parasitic worms, as well as those low types of fungi commonly spoken of as the "yeasts" and "yeast-like organisms," the somewhat indefinite group called "molds," and the extremely numerous and widespread group of the bacteria. The modern general use of the term "protozoology," which has resulted from the many careful studies of the lowest group of animal organisms, has to a great extent in recent years transferred the interest and extended descriptions of many of the types of the animal group of Protozoa from the general field of microbiology to this more specialized domain of study. A modern tendency apparently is to confine the term "microbiology" largely to organisms that fall within the realm of the lowest groups of botanical or plant life. It is in this sense that the word is used in this book.

For several decades it has been known that numerous kinds of yeasts, molds and other low fungi, and several types or groups of bacteria have direct relation, either favorable or unfavorable, to certain types of economic processes carried out in connection with industrial or factory operations such as brewing, wine making, cheese making, etc., which have grown up from small-scale or family arts. Knowledge in this field has greatly extended in recent years, as research in many countries and in many lines has been carried out. The scale of operations has greatly nlarged and concentrated in manufacturing plants. Industries have eplaced household arts. These changes have made it evident not only hat industrial microbiology is an exceedingly interesting branch of study ut that it has already become a distinctly important branch of applied cience, and one with even greater potentialities.

In any broad-gauge discussion of industrial microbiology it should be mphasized at the outset that knowledge in this field is of usefulness and significance from two diametrically opposite angles of application. On the first and more positive side, it is clearly advantageous to know the biological and biochemical characteristics of the many types of organisms that are the prime and direct causes of chemical transformation of materials into desired products. Here one utilizes the microorganisms capable of producing, by fermentation processes, relatively large quantities of chemical substances of usefulness and economic value. In this type of process the biochemical methods are in some instances the only feasible methods of production. Examples are the making of itaconic, kojic and lactic acids, dextran, certain vitamins, and most antibiotics.

Of equal interest to the microbiologist is the part played by organisms of these groups in composite operations, where the action sought is the production of somewhat smaller quantities of desirable by-products that should occur only as minor but important components in the final product. This is the aim in some branches of food manufacturing and in other commodity products.

On the other hand, it is recognized that fermentations incited by microbes may not always be desirable but may be quite the reverse. Competition by invading contaminants may wreck what would otherwise be a successful process. It is, then, of much importance to have knowledge of the organisms specifically useful in a process, and to be equally informed regarding those which oftentimes are destructive to or detrimental in manufacturing processes and are the causes of economic loss. The trained industrial microbiologist must be able to discover them, to recognize the type of damage they produce, and to become versed in the methods of combating them.

With all these viewpoints in mind, it is clear that industrial microbiology should include in its scope the study (1) of the numerous fermentation processes in which the production of alcohols, organic acids, glycerol, acetone, and other substances are end products; (2) of certain aspects of food-manufacturing processes, such as baking and the making of cheese, butter, sauerkraut, and pickles, in which microbic agencies take a significant and important accessory part; (3) of food-conservation methods, such as canning and preserving, refrigeration, quick freezing, and drying, where sterilization or inhibition is imperative; and (4) of the microbiological problems concerned with textile and commercial fibers. Obviously in the processes grouped under (2) and (3) and in a part of those under (1) industrial microbiology is intimately associated with food technology. The work of the industrial microbiologist may also deal with the production and technical uses of bacterial and fungus enzymes, such as amylases and proteases, or with the preservation or protection of wood and the processes of commercial disinfection, wherein the application of suitable chemicals to restrain or prevent fungus, bacterial, or enzyme activity on walls and floors or on materials undergoing processing in the mill or manufacturing plant may make the difference between success and failure of operation.

The production of sera, vaccines, and other therapeutic agents commercially, although generally regarded as in the field of public health or medicine, and hence not here treated, might in reality be considered as a group of processes in industrial biology. It has seemed best to omit these processes in this volume, and also not to include the study of soil microbiology, the use of nitrogen-fixing bacteria, and the study of the phytopathology of economic crop plants, use of disinfectant or inhibitive sprays, etc. Although all these aspects of microbiology are extremely important in agriculture and therefore constitute a part of economic microbiology, they do not fall within the limits of this work.

In the present volume, industrial microbiology will be regarded essentially as the science and art of investigating and controlling technical fermentations, that is, of using microorganisms as reagents to produce desirable end products having possible or well-defined industrial uses and applications. Obviously, conditions in which losses caused by contaminations or wholesale microbic infection affect manufacturing operations must enter into the subject matter. Although all the minute details of particular industries cannot be dealt with in a text of this scope, an attempt is made to present the principles and the general methods of operation. In addition to the consideration of the organisms and biochemical reactions concerned in the better-known industrial fermentations now in use, a few fermentations of present theoretical interest but perhaps potentially capable of technical development are also discussed.

It is important not to make too broad assumptions regarding industrial uses of microbes and to keep in mind the special qualifications which must be possessed by organisms in order that they may be economically utilized in the direct industrial production of materials having distinct commercial value. Obviously the number of species having this valuable property must be limited. The industrially important microbes may be characterized as having at least three outstanding qualities:

1. The ability to grow rapidly in suitable organic substrates and to be easily cultivated in large quantity.

2. The ability to maintain physiological constancy under these conditions and to produce the necessary enzymes readily and profusely, in order to bring about the desired chemical changes.

3. The ability to carry out these transformations under comparatively simple and workable modifications of environmental conditions, and, since the reactions are exothermic, without the application of large quantities of external energy.

Obviously, industrial operations with microbes are more complicated than laboratory experiments but involve the same principles. Large-scale operations must include protection from contamination, and the chemical engineering equipments must be carefully designed to meet the special conditions required in each type of fermentation.

Hundreds of microbes can grow rapidly in solutions of organic substances without yielding significant quantities of valuable products. They may bring about decompositions that conform to the broad general definitions of a fermentation that will be set up, but they may not be productive of a technically important or industrial fermentation.

**Microorganisms of Fermentation.** As has been intimated, the microorganisms of fermentation include yeasts, molds, and bacteria. These microorganisms are unable to manufacture their own food by the ordinary process of photosynthesis since they lack chlorophyll and are classified as fungi, belonging to the phylum Thallophyta. Certain of the Ascomycetes (sac-fungi) and of the Phycomycetes (alga-like fungi), and a large number of species of the bacteria are the principal microorganisms that are directly concerned. A few of the Basidiomycetes are of significance in special types of breakdown of wood and fibers and for the propagation of mycelium potentially important for its food value.

The microorganisms of fermentation differ widely in respect to morphology, size, reaction to free oxygen, manners of reproduction, growth requirements, ability to assimilate or ferment raw (natural) substances, and in other ways. But they are similar in that they are "colorless" and grow most actively in darkness or diffused light, and all produce enzymes by which they catalyze the reactions ascribed to them.

**Variation in Strains.** Even in a so-called "species" there may be a large number of types or strains, and, even in a so-called "pure strain," variation may occur under different environmental and nutritional conditions. Although the usual mode of increase by asexual reproduction favors the constancy of a given species, many factors may tend to cause changes in the chemical composition of a microorganism, the type and quantity of end products formed, and the rate of growth and reproduction. For example, the nature and quantity of the nutrient substances supplied, the temperature of incubation, the reaction of the medium, the oxygen relationships, the presence or absence of stimulating or inhibiting substances in the medium, and various other factors must be controlled and made similar in order to obtain analogous results with the same or related strains of a microorganism. The importance of mere traces of a substance cannot be too much stressed in some instances. Some of the apparent differences in results obtained in research in different laboratories with the supposedly same strain of organism may disappear when conditions of culture and the chemical composition of the media become exactly or essentially the same. It is, however, probably impossible to maintain perfect lack of variation over long periods.

Although some differences are only apparent ones, frequently true biologic variations exist in strains. Too much work has been carried out by responsible laboratories to leave any doubt about this fact. For example, some strains of a microorganism apparently require added growth-accessory substances, while other strains require none; some strains of *Aspergillus niger* are stimulated by iron and zinc salts, and others receive no apparent stimulation from these salts.

**Fermentation.** From the biochemical standpoint, "fermentation" is the name given to the general class of chemical changes or decompositions produced in organic substrates through the activity of living microorganisms. Thus there may be many kinds of fermentation falling within this category depending on the type of organism involved, the type of substrate, or even the conditions imposed, such as pH, or oxygen supply. The word "fermentation" is a term that has undergone numerous changes in meaning during the past hundred years. According to the derivation of the term, it signifies merely a gentle bubbling or "boiling" condition, and the term was first applied when the only known reaction of this kind was in the production of wine. Even then no knowledge existed about the cause. Thus in an active ethyl alcohol fermentation, as in a wine or cider fermentation, carbon dioxide is always liberated in the form of bubbles of gas, which at the height of the reaction may cause a marked agitation or movement of the liquid medium, especially in a large vat or tank, sufficient to give to it the appearance of a boiling liquid. This interpretation of the word was the accepted one for several thousand years. After Gay-Lussac studied the process the meaning was changed to signify the breakdown of sugar into alcohol and carbon dioxide. With the increase in knowledge following Pasteur's researches into the *cause* of this change in the nature of the material fermenting, the word became associated with microorganisms and still later with enzymes, which are the biologically produced reagents by which microbes work. For a long time fermentation was especially associated with carbohydrates and, indeed, is often so considered at present, but a broader conception of these biologic reactions seems to be more logical. Thus putrefaction and the breakdown of fats by microbes are to be regarded as special kinds of fermentations.

Although fermentation is frequently or even generally associated with the evolution of gas caused by the action of living cells, neither gas evolution nor the visible presence of living cells is today regarded as an essential criterion of fermentation. In certain fermentations, for example, some of the lactic fermentations, no gas is liberated. Again, fermentation may result (although it is not frequently the case) from the use of cell-free enzyme extracts that for a time catalyze the reactions. Gas can be liberated in some of these processes, as, for example, with a zymase preparation.

Fermentations are so varied in character that any broad definition that will cover this whole class of biochemical processes must be couched in very general terms. Nevertheless, it is sometimes useful to gain a briefly stated concept of such a group of reactions. The idea might be expressed as follows:

A "fermentation," in the broad sense in which the term is now used, may be defined as a process in which chemical changes are brought about in an organic substrate through the action of enzymes elaborated by microorganisms. Although fermentation generally takes place in the presence of the living cells, it may, in some cases, occur in their absence, for example, in the production of dextran.

Carbohydrates are often regarded as being essential materials for fermentation, but it must be clear that organic acids (including amino acids) and their salts, proteins and their derivatives, fats, sterols, alcohols, esters, and other organic compounds are fermentable under certain conditions by selected microorganisms.

Industrial fermentations are those in which chemical products of value are manufactured.

**Propagation.** The term "propagation" should be used to denote those processes wherein the primary objective is to produce cells rather than end products. Bakers' yeast, food yeast, mushroom mycelium, and inocula are examples of items produced by propagation processes. Aeration favors the development of cells of aerobic species. Enzymes are involved in the assimilation of food materials and in the biosynthesis of protoplasm.

Fermentations may be associated with propagation processes but may be largely prevented or minimized by maintaining optimum conditions for the reproduction of the cells.

**Enzymes.** The student of biology or biochemistry will already have learned of the universal association of enzymes with living matter and the important part played by them in all types of vital activity. The activities of enzymes are especially prominent in the phenomena of digestion in animals, in the transformations of starch in sprouting seeds, etc. These and many other observations lead to the view that enzymes are the essential organic catalysts, possessed or produced by all living cells, without which the processes of life would cease or be impossible. It is, furthermore, well established that these enzymes are of two types, called "exoenzymes" and "endoenzymes," according to their sphere of activity outside or within the confines of the cells that elaborate them. Both types are extremely important. Exoenzymes, liberated by the manufacturing cells, penetrate and break down the organic materials outside the cell, such as the proteins, starches, and fats of food materials, giving rise to soluble derivatives and so making it possible for the products of their activity to be absorbed through the cell membrane. Energy liberated by the enzyme action outside the cell is of comparatively little value to the cell. In general it is found that exoenzymes liberate relatively little energy, as heat, especially in the most commonly produced hydrolytic processes.

The endoenzymes elaborated and retained within the living cell, on the other hand, behave quite differently. The food substances, having been absorbed into the cell, may be further transformed and broken down by the action of the endoenzymes, and this process is accompanied by the liberation of relatively large amounts of energy, this energy being available to the cell.

The microorganisms of fermentation are notable for their ability to produce enzymes of both these classes. Molds, yeasts, and bacteria can secrete or elaborate a wide variety of enzymes, possibly of a greater range than any other single cells, since all the functions of growth, reproduction, digestion, assimilation, etc., that are distributed among the various tissues and organs of higher plants and animals seem here to be concentrated in the single minute cell. This may explain why it is that, in these low groups of extremely small plants which are essentially unicellular in structure, we find a higher degree of enzyme productivity and of fermentative capacity than is exhibited elsewhere in the world of living things.

The enzymes are therefore the reactive substances or catalyzers that microorganisms employ in bringing about the specific changes or fermentations that are characteristic of different species or groups of microbes. One can visualize each strain, species, or genus as having its own armamentarium of enzymes or its own peculiar ability to secrete them. In this individual potentiality may lie one of the factors that account for the variations in strains mentioned earlier in this chapter.

**Enzyme Production.** Microbes are used to produce industrially important enzymes. The processes in which the enzymes are formed are often closely related to propagation processes. The enzymes are elaborated by methods which are designed to obtain a maximum number of units of enzyme activity. There is a direct relationship between the number of cells and the enzyme activity under a given set of conditions. However, the conditions which favor the maximum yield of cells are by no means those which favor the maximum yield of enzyme activity (unless by coincidence).

**Energy Relationships.** Table 1, which shows the energy liberated from 1 g. of substrate by enzymes, will give some insight into the relative energy values of exoenzymes and endoenzymes.

TABLE 1. ENERGY LIBERATED FROM 1 GRAM OF SUBSTRATE BY ENZYMES *

| Exoenzymes | Calories | Endoenzymes | Calories |
|---|---|---|---|
| Pepsin | 0 | Lactacidase | 82 |
| Trypsin | 0 | Alcoholase | 149.5 |
| Rennet | 0 | Urease | 239 |
| Lipase | 4 | Oxidase (vinegar) | 2,530 |
| Invertase | 9.3 | | |
| Maltase | 10 | | |
| Lactase | 23 | | |

* C. G. Anderson, "An Introduction to Bacteriological Chemistry," The Williams & Wilkins Company, Baltimore, 1938.

The question naturally arises, Does the microorganism have a requirement for a major portion of the large amounts of energy liberated within the cell, or is much of the energy liberated merely due to the great excess of enzyme product or the result of unrestricted enzyme action? Experiments with yeast cells indicate rather emphatically that the energy evolved is not a measure of metabolic requirements but is the result of enzymes acting on an abundant and suitable substrate. However, further experimentation is necessary to lend additional weight to this suggestion. Whatever the theoretical aspects, it is known in practice that under the artificial conditions of a great volume of fermenting liquid, in which billions of cells are active, energy in the form of heat may be liberated to a marked degree. In such large masses of fermenting material where the heat produced cannot be readily diffused or conducted away the rise of temperature may become so great as to impair the organisms that produce the reacting catalyst. In vinegar fermentation the heat energy evolved may be sufficient to cause a rise of temperature that may inhibit normal cell growth and stop further activity.

**Intense Activity of Microorganisms.** Many microorganisms, in comparison to the higher organisms, are intensely active in respect to the chemical change that they bring about. The examples cited above show this fact and also are typical of the fact that all fermentative processes are exothermic. Burchard[1] has calculated that 1 g. (wet weight) of *Micrococcus ureae* decomposes 180 to 1,200 g. of urea per hour, while Haacke estimated that 1 g. (wet weight) of a lactose fermenting organism breaks down 178 to 14,890 g. of lactose in 1 hr. Even though these calculations may be of only approximate value, the enormous activity of microorganisms is evident. This great chemical activity of microorganisms is associated with their simple life requirements, the ease with which they attack food for energy, the rapidity of growth or reproduction, and possibly their capacity for maintenance under different conditions.

**Specific Types of Fermentative Change.** It has been intimated in the foregoing pages that the specific or characteristic kind of fermentation produced by an organism is dependent on its enzyme-producing powers. One might expect, therefore, that those organisms most nearly related generically would be most similar in their fermentation relationships. This seems to be generally the case, although it does not follow that organisms belonging to different biological groups are necessarily totally unlike in their ability to catalyze fermentation processes having some of the same end products. The "true" yeasts and some other budding fungi nearly related to them are the organisms that most commonly produce ethyl alcohol, and because of this fact it is often stated that the yeasts are the microorganisms of alcoholic fermentation; but there are many budding fungi, classified in groups or families morphologically very similar to yeasts, that apparently have no ability to produce ethyl alcoholic fermentation or any other with large quantities of a particular end product. On the other hand there are a few fungi, somewhat higher in the scale of organization than yeasts, that can produce ethyl alcohol under certain conditions of substrate, pH, relation to oxygen, etc. Even a very small number of species of bacteria can produce alcohol. These are unusual cases but apparently demonstrate the great versatility of the low, colorless plants in developing their enzyme systems. In general, the molds that have marked fermentation power are producers of organic acids and of products of protein decomposition. Similarly, the fermenting types of bacteria commonly give rise to organic acids, frequently different from the acids produced by molds, and to higher alcohols than ethyl. It cannot be assumed that all species of microorganisms belonging to the three groups that have been mentioned as constituting the microorganisms of fermentation actually have marked ability in this respect. It would be more nearly correct to say that the significant fermentation organisms represent a minority of all the organisms so classed.

---

[1] M. Stephenson, "Bacterial Metabolism," Longmans, Green & Co., Ltd., London, 1930.

**Energy Value of Substances.** The substances acted on by microorganisms present a dual role, the first as a food, the second as a material to be transformed through the action of enzymes that have been produced in excess of nutritional requirements. In general, the energy value of a substance, such as glucose, depends on the degree of oxidizability it can undergo. In the complete oxidation of glucose, as in burning, considerable energy is evolved. This is also true in the breakdown by aerobic organisms, while in the anaerobic breakdown of this sugar only a fraction of the potential energy is liberated. Consequently, in order to obtain an equivalent amount of energy, several times as much glucose must be broken down under anaerobic conditions as would be required under aerobic conditions.

The breakdown of nutrients in fermentation is not merely expressed in the evolution of heat but is in early stages at least always accompanied by division of new cells. For example, in the manufacture of compressed yeast, the nutrient medium is supplied not only with organic food but also with a very large amount of air. Aerobic oxidations are favored and under such conditions, along with the fulfillment of nitrogen and phosphate requirements, pH control, and the employment of a low concentration of sugar, large yields of yeast cells are produced. Under essentially anaerobic conditions, such as would exist in the normal industrial fermentation for ethyl alcohol, much more sugar must be utilized to produce the same quantity of yeast cells, and a large amount of the organic matter of the substrate is converted to the characteristic end products, one of which, ethyl alcohol, is still comparatively high in potential energy.

It should now be clear that a fermentation process that may become of industrial significance is not a simple matter of inoculating organic matter with microbes that can derive food from it, but is instead the setting up of a system of biochemical reactions between a substrate and an organism that can partially decompose it in a special manner under controlled and workable conditions. The substrate must be abundant, inexpensive, and fairly high in potential energy. The organism must have ability to attack, by means of its enzymes, certain chemical groupings in the composition of the substrate and, by hydrolysis, oxidation or reduction, or other means, disrupt certain bonds in the substrate molecules and give rise to stable products under the imposed conditions. Thus by a single reaction or a series of coincident or successive enzyme reactions carried out under definite conditions it may bring about eventually a desired product or group of products of lower potential energy.

# CHAPTER 2

# THE YEASTS

The yeasts and yeast-like organisms belong to the subdivision of the thallophytes designated as the Eumycetes, or true fungi, since they possess no chlorophyll.

Yeasts are grouped together in three families, known as the Saccharomycetaceae, the Sporobolomycetaceae, and the Cryptococcaceae. They represent a somewhat poorly defined group of microorganisms which lies between the bacteria and higher fungi in respect to the average size of the single cell. The group is characterized by a form of vegetative reproduction known as "budding." However, budding and/or fission may occur in some species. Although budding cells may always be found in the Cryptococcaceae, mycelium, pseudomycelium, and arthrospores may be formed by some species of yeasts. Thus it is not possible to characterize yeasts as microbes, which always occur as single cells.

Table 2 shows the relationship of the groups of yeasts to the classes of fungi and presents the diagnosis of each family as given by Lodder and Kreger-van Rij (1952).

The number of genera and species of yeasts varies, depending on the classification concerned. In the classification of Lodder and Kreger-van Rij (1952, 1954), there are 16 genera, including about 71 species listed in the family Saccharomycetaceae, 2 genera and 9 species in the family Sporobolomycetaceae, and 9 genera and 87 species in the family Cryptococcaceae. Mrak (1957) states that the number of genera and species would be larger with a broader and more inclusive definition of the term "yeast."

**Occurrence and Distribution of Yeasts.** The budding fungi are of wide distribution in nature. Their habitats may include not only the upper layers of the soil but also many forms of organic matter, especially of plant origin, where carbohydrates are of common occurrence. Yeasts may be isolated particularly from the soil of vineyards and orchards; from the surfaces of grapes, apples, and most sweet fruits; from citrus fruits; and from the leaves and other parts of plants. They are carried into the air with dust and on the bodies of insects and thus may be widely disseminated. Proctor (1934) has shown that yeasts may be found in the air at high altitudes. Some types of yeasts are also found occasionally on animal products.

Table 3 shows the predominating types of yeasts found in some selected sources by various scientists.

TABLE 2. DATA CONCERNING THE YEASTS

| Ascosporogenous yeasts | Ballistospore-forming yeasts | Asporogenous yeasts |
|---|---|---|
| Class: Ascomycetes<br>Order: Endomycetales<br>Family: Saccharomycetaceae | Class: Basidiomycetes or Fungi imperfecti<br>Family: Sporobolomycetaceae | Class: Fungi imperfecti<br>Order: Cryptococcales<br>Family: Cryptococcaceae |
| *Diagnosis* * | *Diagnosis* * | *Diagnosis* * |
| "Mycelium, pseudomycelium, arthrospores, and budding cells side by side or alone. Vegetative reproduction by fission or by budding. Asci may arise after isogamous or heterogamous conjugation; often a stage of vegetative reproduction between diploidization and ascus formation. Ascospores of various shapes. They may conjugate. Besides an oxidative dissimilation a fermentative dissimilation is often present." | "Mycelium, pseudomycelium, and budding yeast cells. Vegetative reproduction by fission or by budding. Part of the vegetative cells form aerial sterigmata either singly or rarely bifurcated. Smooth, hyaline spores are formed which develop in an oblique position to the sterigmata. When mature, the spores are ejaculated by a drop-excretion mechanism (ballistospores). Dissimilation strictly oxidative." | "Budding cells aways present; moreover, mycelium, pseudomycelium and arthrospores may be formed. Reproduction by budding; besides fission may occur. Cells hyaline, never dark or brown; carotenoid pigments may be present. Dissimilation strictly oxidative or besides oxidative also fermentative." |

---

* Quoted by permission from J. Lodder and N. J. W. Kreger-van Rij, "The Yeasts, a Taxonomic Study," North Holland Publishing Co., Amsterdam, 1952.

Table 3. Predominating Types of Yeasts Found in Some Selected Sources

| Source | Predominating types | Reference |
|---|---|---|
| Commercial meat brines | *Debaryomyces membranaefaciens* var. *Hollandicus*, *D. klockeri* Guill. et Péju | Costilow, R. N., J. L. Etchells, and Blumer: *Appl. Microbiol.*, **2**(5): 300–302 (1954). |
| Commercial olive brines | *Candida krusei*, *C. tenuis*, *C. solani*, *Torulopsis sphaerica*, *T. holmii*, *Hansenula subpelliculosa*, *Pichia membranaefaciens*, *C. mycoderma*, *C. rugosa* | Mrak, E. M., R. H. Vaughn, M. H. Miller, and H. J. Phaff: *Food Technol.*, **10**(9): 416–419 (1956). |
| Cucumber brine (film) | *D. membranaefaciens* var. *Hollandicus*, *Saccharomyces* (Zygosaccharomyces) *halomembranis* | Etchells, J. L., and T. A. Bell: *Food Technol.*, **4**: 77–83 (1950). |
| Cucumber brine (subsurface) | *Brettanomyces versatilis*, *T. caroliniana*, *T. holmii*, *Torulaspora rosei*, *H. subpelliculosa*, *Saccharomyces* sp. | Etchells, J. L., and T. A. Bell: *Farlowia*, **4**: 87–112 (1950).<br>Etchells, J. L., R. N. Costilow, and T. A. Bell: *Farlowia*, **4**: 249–264 (1952). |
| Pickle brines | *Debaryomyces*, *Pichia*, *Mycoderma* (*Candida*) | Mrak, E. M., and Bonar: *Zentr. Bakt. Parasitenk., II*, **100**: 289–294 (1939). |
| Cucumber plant | *Rhodotorula*, *Candida*, *Torulopsis*, *Debaryomyces*, *Torulaspora*, *Kloeckera*, *Saccharomyces* | Etchells, J. L., T. A. Bell, and I. D. Jones: *Farlowia*, **4**(3): 265–304 (1953). |

| | | |
|---|---|---|
| Citrus products | *Candida*, *Zygosaccharomyces* (*Saccharomyces*), *Hanseniaspora*, *Saccharomyces*, *Pichia* | Recca and E. M. Mrak: *Food Technol.*, **6**(12): 450–454 (1952). |
| Shrimp | *Rhodotorula*, *Trichosporon*, *Candida*, *Pullularia* | Phaff, H. J., E. M. Mrak, and O. B. Williams: *Mycologia*, **44**(4): 431–451 (1952). |
| *Shiokara* (fishery-fermentation product) | *Debaryomyces*, *Saccharomyces* | Zenitani: *Science Bull., Fac. Agr. Kyushu Univ.*, **12**: 247–253 (1952). |
| Yeast spoilage of English draft beers | *Saccharomyces* | Wiles, A. E.: *Jour. Inst. Brewing*, **56**: 183–193 (1950). |
| Contaminants of brewery yeasts and beers | *C. mycoderma* | Walker, T. K., and A. E. Wiles: *Jour. Inst. Brewing*, **58**(2): 140–145 (1954). |
| Dried fruit beetles in figs | *C. krusei*, *Hanseniaspora valbyensis* | Miller, M. W., and E. M. Mrak: *Appl. Microbiol.*, **1**(4): 174–178 (1953). |
| Intestinal tracts of wild species of *Drosophila* (Yosemite region of California) | *S. montanus*, *S. veronae*, *S. cerevisiae* var. *tetrasporus*, *S. drosophilarum*, *H. angusta*, *K. apiculata*, *K. magna*, *T. stellata*, *C. krusei* | Phaff, H. J., M. W. Miller, and Shifrine: *Antonie van Leeuwenhoek Jour. Microbiol. Serol.*, **22**: 145–161 (1956); *Ecology*, **37**(3): 533–538 (1956). |
| Barkbeetles *Ips* and *Dendroctonus* (northern California) | *H. capsulata*, *S. pini*, *C. silvicola* | Shifrine and H. J. Phaff: *Mycologia*, **48**(1): 41–55 (1956). |
| Natural breeding sites of some species of *Drosophila* | *H. mrakii* (imperfect form), *P. silvestris*, *S. pastori* | Carson, Knapp, and H. J. Phaff: *Ecology*, **37**(3): 538–544 (1956). |

TABLE 4. DATA CONCERNING SOME SPECIES

| Yeast (genus and species) | Shape of cells | Size of cells (3 days at 25°C. on malt agar), μ |
|---|---|---|
| *Schizosaccharomyces octosporus*. | Round or cylindrical | (5–7.5) × (5–11) |
| *Endomycopsis fibuliger*........ | Oval to elongate | (4.2–6.6) × (6–15) |
| *Saccharomyces cerevisiae*....... | Round, short oval, or oval | (3–10) × (4.5–15) |
| *S. carlsbergensis*............. | Round, short oval, or oval | (3.5–8) × (5–15.5) |
| *S. mellis*.................. | Round to oval | (2.5–5.3) × (2.5–7) |
| *Pichia membranaefaciens*...... | Oval, long oval, or cylindrical | (2.5–4.5) × (5–14) |
| *Hansenula subpelliculosa*...... | Round to oval | (2–5) × (3.5–10)[a] |
| *Schwanniomyces occidentalis*.... | Oval or egg-shaped | (3–5.5) × (4.5–9) |
| *Debaryomyces hansenii*........ | Round to short oval | (2.2–5) × (3.4–6.5) |
| *Saccharomycodes ludwigii*...... | Lemon- or sausage-shaped | (4–8.5) × (9–36) |
| *Hanseniaspora valbyensis*...... | Lemon-shaped, oval, or long oval | (1.5–4.5) × (4–10) |
| *Nadsonia elongata*............ | Oval to elongate or lemon-shaped | (4–7) × (7–15) |
| *Nematospora coryli*........... | Nearly round to wide oval, or slender and elongate | (2.5–8) × (5–17) |
| *Lipomyces starkeyi*............ | Round to short oval | (4–6.5) × (5–8) |

* Compiled from J. Lodder and N. J. W. Kreger-van Rij, "The Yeasts, a Taxonomic Study," North Holland Publishing Co., Amsterdam, 1952.

CODE:
- [a] Occasionally weak.
- [b] Very weak.
- [c] Weak.

OF THE FAMILY SACCHAROMYCETACEAE *

| Method of vegetative reproduction | Fermentation | | | | | | Assimilation | | | | | KNO$_3$ assimilation | Ethanol as carbon source | Arbutin splitting |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose | Galactose | Sucrose | Maltose | Lactose | Raffinose | Glucose | Galactose | Sucrose | Maltose | Lactose | | | |
| Fission | + | − | −[a] | + | − | −[a] | + | − | −[b] | + | − | − | − | − |
| Budding and fission | +[c] | − | +[c] | +[c] | − | +<br>1/3[d] | + | − | + | + | − | − | + | Variable |
| Budding (multilateral) | + | + or<br>−[a] | + | + | − | +<br>1/3 | + | +[a] | + | + | − | − | [e] | − |
| Budding (multilateral) | + | +[a] | + | + | − | + | + | + | + | + | − | − | − | − |
| Budding (multilateral) | + | − | − | − | − | | + | + or<br>− | − | − | − | − | − | − |
| Budding (multilateral) | −[f] | − | − | − | − | − | + | − | − | − | − | − | + | − |
| Budding (multilateral) | + | − | + | + or<br>−[c] | − | +<br>1/3 | + | + or<br>−[c] | + | + | − | + | +[a] | + |
| Budding (multilateral) | + | − | + | − | − | +<br>1/3 | + | + | + | + | − | − | − | +[c] |
| Budding (multilateral) | +[h] | + or<br>−[h] | +[h] | + or<br>−[h] | | | + | + | + | + | +[a] | − | + | +[f] |
| Budding (at poles) | + | − | + | − | − | +<br>1/3 | + | − | + | − | − | − | − | +[c] |
| Budding (bipolar) | + | − | − | − | − | | + | − | − | − | − | − | − | − |
| Budding (bipolar) | + | − | − | − | − | | + | − | − | − | − | − | + | − |
| Budding (multilateral) | + | − | +[a] | +[i] | − | +[b] | + | ? | + | + | − | − | − or<br>+[b] | − |
| Budding (multilateral) | − | − | − | − | − | − | + | + | + | + | − | − | +[i] | +[i] |

[d] Or less.
[e] Usually no growth, but occasionally weak growth.
[f] Occasionally very weak.
[g] Occasionally longer.
[h] Very slight.
[i] Often weak.

**Shapes of Cells.** The individual yeast cells are usually spherical, ovoid, or ellipsoid in form. However, broadly egg-shaped, sausage-shaped, and lemon-shaped cells are produced characteristically by some species of yeasts. The shape of the active cell is not an exact means of species identification, nor is variety in form in the same culture a proof of contamination. Yeasts do not possess flagella, and consequently the individual cells are nonmotile. Figure 1 illustrates a few of the different kinds of yeast cells that occur in nature.

**Size.** Yeast cells may vary considerably in dimensions, depending on the species, the nutrition, the age, and other factors. The usual cell may vary from a little over 1 to 9 or more microns in width and from about

TABLE 5. DATA CONCERNING SOME

| Genera and species of Cryptococcaceae | Shape of cells | Size of cells (3 days at 25°C. on malt agar) |
|---|---|---|
| *Cryptococcus neoformans* | Round or almost round | (2.5–7) |
| *Torulopsis lactis-condensi* | Oval to long oval | (1.3–2.5) × (1.9–6) |
| *Pityrosporum ovale* | Oval or bottle-shaped | (2–3) × (3.8–5.8)[b] |
| *Brettanomyces bruxellensis* | Usually oval, also round or elongate | (2.5–4) × (3–9)[c] |
| *Candida tropicalis* | Short oval to oval, occasionally round | (5–9) × (6–12)[f] |
| *C. utilis* | Oval | (3–5.5) × (5–9)[f] |
| *C. lipolytica* | Long oval to elongate, also short oval | (2–4.5) × (4–22)[f] |
| *Kloeckera apiculata* | Lemon-shaped, short oval, oval, or long oval | (2–4.5) × (5–10)[f] |
| *Trigonopsis variabilis* | Triangular or ellipsoidal | (3.0–5.0) × (3.5–5.0) |
| *Trichosporon fermentans* | Cylindrical or elongate | (4–8) × (10–20)[f] |
| *Rhodotorula glutinis* | ........................... | (2.5–5.5) × (5–10) |

[*] Compiled from J. Lodder and N. J. W. Kreger-van Rij, "The Yeasts, a Taxonomic Study," North Holland Publishing Co., Amsterdam, 1952.

CODE:

[a] Occasionally weak.
[b] After 5 days.
[c] After 5 days in malt extract.

2 to 20 or more microns in length. With the spherical cells of industrial yeasts a diameter of 4 to 6 microns is probably a fair average, but great variations may occur even in the same culture. Most of the yeasts of extremely small size are of no present industrial importance except as they occur as contaminants.

In general, yeast cells are much larger than bacterial cells and could not be mistaken for the latter when observed microscopically. There are exceptions, however; some large bacteria exceed in size the smallest known yeasts mentioned in the figures given.

Tables 4 and 5 contain data on the sizes of representative species of yeasts.

SPECIES OF CRYPTOCOCCACEAE *

| Fermentation | | | | | | Assimilation | | | | | KNO$_3$ assimilation | Ethanol as carbon source | Arbutin splitting |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Glucose | Galactose | Sucrose | Maltose | Lactose | Raffinose | Glucose | Galactose | Sucrose | Maltose | Lactose | | | |
| − | − | − | − | − | − | + | +[a] | + | + | − | − | + | + |
| + | − | + | − | − | +⅓ | + | − | + | − | − | +[a] | − | − |
| − | − | − | − | − | − | | | | | | | | |
| + | − | + | + | − | −[d] | + | +[e] | + | + | − | + | + | |
| + | + | + | + | − | +[f] | + | + | + | + | − | − | −[g] | −[h] |
| + | − | + | − | − | +⅓ | + | − | + | + | − | + | + | + |
| − | − | − | − | − | − | + | − | − | − | − | − | −[g] | −[h] |
| + | − | − | − | − | − | + | − | − | − | − | − | +[i] | − |
| − | − | − | − | − | − | + | + | − | − | − | − | +[j] | − |
| + | + | − | − | − | − | + | + | − | − | − | − | + | − |
| − | − | − | − | − | − | + | + | + | + | − | + | + | + |

[d] Weak fermentation may occur after 8 days or longer.

[e] Weak growth or no growth may also occur.

[f] After 3 days in malt extract.

[g] No growth or very little growth.

[h] Absent or slightly positive.

[i] Scarcely any growth.

[j] Weak growth.

**The Cell Wall.** This part of the yeast cell covers and protects the cytoplasm and the nuclear material. It is permeable to some substances and impermeable to others. Its exterior is the site of activity of surface-active and other compounds.

The cell wall of *Saccharomyces cerevisiae* and other yeasts has been studied recently by a number of scientists. Roelofsen and Hoette (1951) found that chitin was a constituent of the cell walls of a number of ascosporogenous and anascosporogenous species of yeasts, although the amount was very small and varied in different species. Houwink and Kreger (1953), using an electron microscope and X-ray diffraction study, observed yeast glucan and much smaller amounts of chitin in the cell walls of bakers' yeast and *Candida tropicalis*. Bartholomew and Mittwer (1953) demonstrated the presence of bud scars on cell walls, with the aid of the electron microscope. Northcote and Horne (1952), using chemical and microscopic methods, reported that the cell wall contained at least two distinct layers. Agar and Douglas (1955) confirmed the presence of several distinct layers in the cell wall of the tetraploid strain of *S. cerevisiae* and of bud scars (using the electron microscope to study thin sections).

**Contents of the Cell.** The protoplasm appears microscopically as composed of a grayish, finely granular, semifluid mass. Presumably it is of albuminous material intimately mixed with a "cell sap" of water with organic materials and salts in solution. Within the protoplast there are a nucleus (Fig. 2) and one or more rounded structures known as

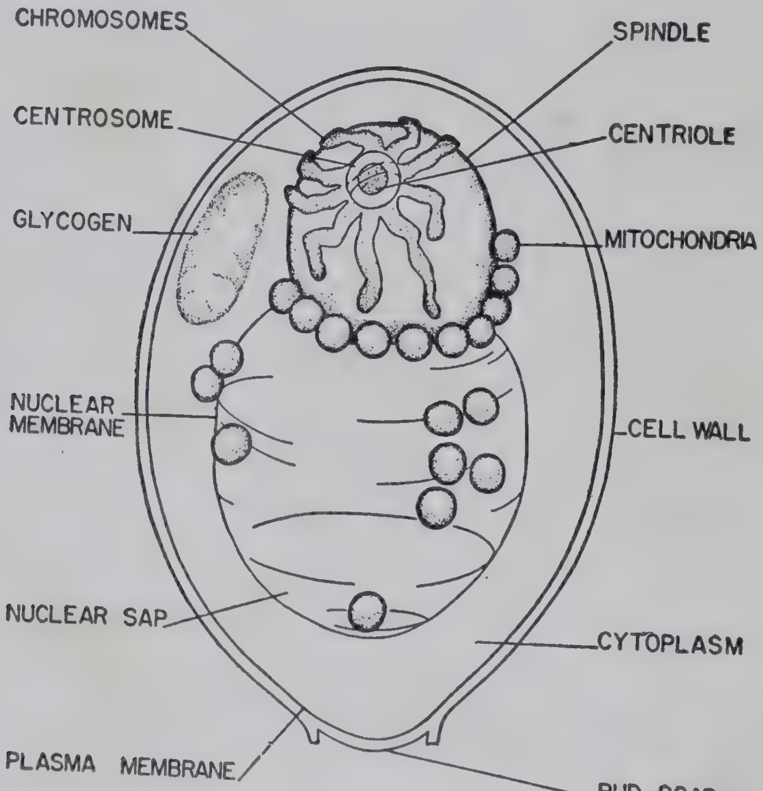


Fig. 2. A diagrammatic representation of the dormant yeast cell. [*Courtesy of C. C Lindegren, Antonie van Leeuwenhoek Jour. Microbiol. Serol.,* 22: 1 (1956).]

lered

## NEMATOSPOROIDEAE

Mycelium and budding cells
Iso conjugation; needle or spindle-shaped spores, with or without appendages
Oxidative or fermentative

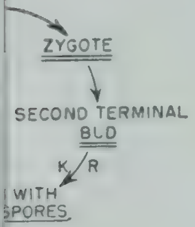
## LIPOMYCETOIDEAE

Budding cells, round to oval
Sac-like ascus with protuberance of a vegetative cell
Spores oval, amber; usually with 4 or 8, occasionally 16 or more per ascus
Cells surrounded by a capsule of slime
Life cycle uncertain
Oxidative only

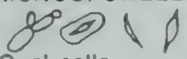
m

lm) and
ve
n shaped

gation of
l and bud;
d forms,
an ascus
warty spores

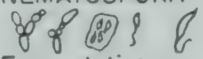


### MONOSPORELLA



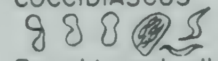
Oval cells
Budding
Iso conjugation
Only 1 needle-shaped spore

### NEMATOSPORA

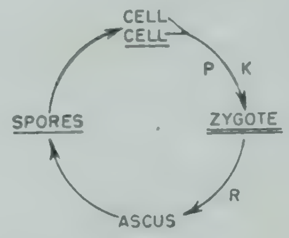


Fermentative
Oval, long, irregular or mycelium-like cells
No apparent conjugation
Usually 8 spindle-shaped spores with appendage

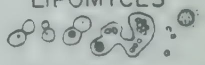
### COCCIDIASCUS



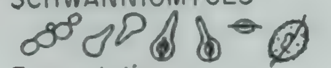
Round to oval cells
Iso conjugation
8 spindle-shaped spores



### LIPOMYCES

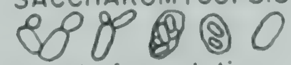


### SCHWANNIOMYCES



Fermentative
Oval cells
Retort shaped ascus
Abortive conjugation tubes
One (seldom 2) round, warty spores with ledge

### SACCHAROMYCOPSIS



Weakly fermentative
Ellipsoidal to long oval cells, budding
Double-walled spore, oval to elongate, one to 4 per ascus

NOTE:

R....MEIOTIC OR REDUCTION DIVISION
P....PLASMOGAMY, FUSION OF PROTOBLAST OF CONJUGATING CELLS
K....KARYOGAMY, FUSION OF NUCLEI OF CONJUGATING CELLS

SINGLE UNDERLINE.....HAPLOID GENERATION

DOUBLE UNDERLINE.....DIPLOID GENERATION

*'alifornia, Davis, Calif.)*

"vacuoles," which were originally so called because they appeared as clear or empty spaces but are now known to be the seat of reserve foods and finely divided "metachromatic granules" or volutin. The vacuoles are not conspicuous in very young cells but appear prominently in mature or old cells, and their nature can be somewhat determined by special staining methods.

Within the yeast cell protoplasm are numerous other substances or reserve materials in the form of droplets or granules, some of which are exceedingly complex in nature. In old cultures, some cells sometimes become thick walled and much enlarged and densely packed with these reserves. Such cells are sometimes called "durable" or "durative" cells, as they seem to have unusual resistance to adverse conditions.

These stored foods—including some carbohydrates (not starch), fats, or oils as refractile droplets, and some granules supposed to be proteins—are normal constituents of all mature yeast cells but especially of the older cells, when nutritive conditions are favorable. As stated, this reserve food is found dissolved in the cell sap in vacuoles and in the form of granules or droplets.

**Yeast Spores.** Sporulation in yeasts is important for three reasons: it is the basis of a method of reproduction, it performs a function in the making of new hybrids, and it serves a role in maintaining the viability of the species during adverse changes in the environment. The ascospores of yeasts are somewhat more resistant to heat and desiccation than the vegetative cells, yet they are much less resistant to heat than the spores of many bacterial species. In general, they are also less resistant to cathode and X rays than bacterial spores.

**Sporulation.** Sporulation may be initiated by a deficiency in the food supply and the accumulation of toxic end products but will not proceed unless certain other conditions are favorable; namely, the yeast cells must be young and vigorous, there must be plenty of air and moisture available, the pH of the medium must be suitable, inhibitory substances must be absent, and the temperature of incubation must be satisfactory. The presence of stimulating substances promotes sporulation.

In order to produce vigorous young cells, the yeast should be cultured in a suitable medium and transferred frequently. Reserve foods—glycogen, fat, and other products—are stored in the yeast. The use of such cells will ensure sporulation, provided the other conditions are satisfactory and the yeast has the ability to form endospores.

Oxygen is essential for sporulation; without it no spores are formed.

Temperature is important. Each variety of yeast sporulates most readily in a given temperature range. Above certain temperatures and below others, sporulation fails to take place. Table 6 shows the maximum, minimum, and optimum temperatures for the sporulation of six varieties of yeasts studied by Hansen. Table 7 gives data concerning scospores.

The optimum temperatures for the six varieties lie between 25 and 0°C., while three varieties have an optimum temperature of 25°C. At

TABLE 6. MAXIMUM, MINIMUM, AND OPTIMUM TEMPERATURES FOR SPORULATION OF CERTAIN YEASTS *

| Yeast | Maximum temperature, °C. | Minimum temperature, °C. | Optimum temperature, °C. |
|---|---|---|---|
| *Saccharomyces cerevisiae* | 35 –37 | 9 –11 | 30 |
| *S. pastorianus* | 29 –31.5 | 0.5– 4 | 27.5 |
| *S. intermedius* | 27 –29 | 0.5– 4 | 25 |
| *S. validus* | 27 –29 | 4.8– 5 | 25 |
| *S. ellipsoideus* | 30.5–32.5 | 4.7– 5 | 25 |
| *S. turbidans* | 33 –35 | 4 – 8 | 29 |

* Reprinted by permission from A. Guilliermond, "The Yeasts," translated an revised by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

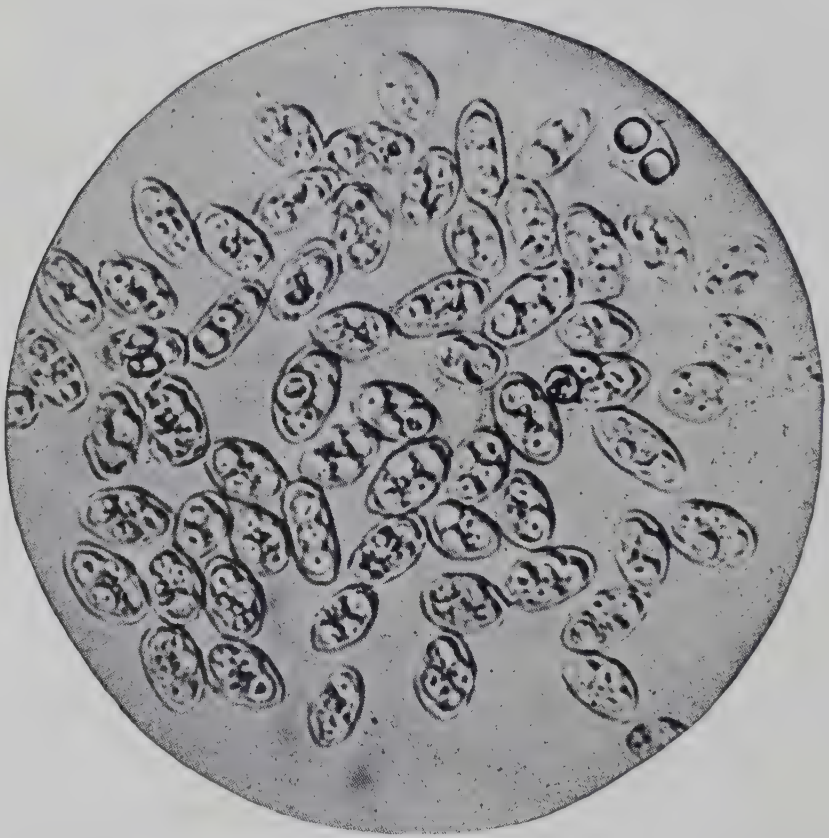


FIG. 3. Sporulation of yeast. (*Courtesy of H. Kothe and F. R. Swift, Fleischman Laboratories.*)

the most favorable temperature for sporulation, ascospores begin t appear in 21 hr. or more.

Calcium sulfate stimulates sporulation but restrains budding. Bee containing glucose or other sugars or gelatin stimulates sporulation, bu ammonium salts have an adverse effect. Green, blue, violet, and ultra violet rays have inhibitory effects on the formation of endospore Extremes of pH definitely retard sporulation.

TABLE 7. DATA CONCERNING ASCOSPORES

| Genus | Shape of spore | Characteristics of spore surface | Usual number of spores per ascus |
|---|---|---|---|
| *Endomyces* | Hat-shaped or oval | Smooth | 4 |
| *Schizosaccharomyces* | Spherical, oval, or kidney-shaped | Smooth | 4 or 8 |
| *Endomycopsis* | Hat-shaped, oval, sickle-shaped, spherical, or Saturn-shaped | Smooth, occasionally with brim | 4 |
| *Saccharomyces* | Spherical to oval | Smooth | 1–4 |
| *Pichia* | Hemispherical, spherical, angular, or hat-shaped | Smooth, occasionally with brim | 1–4 |
| *Hansenula* | Hat-shaped, Saturn-shaped, spheroidal, or hemispheroidal | Smooth with brim on base | 1–4 |
| *Schwanniomyces* | Spherical or walnut-shaped | Warty, equatorial brim | 1 |
| *Debaryomyces* | Spherical | Warty | 1–2 |
| *Saccharomycodes* | Spherical | Smooth | 4 |
| *Hanseniaspora* | Spherical or hat-shaped | Smooth, occasionally with brim | 1–4 |
| *Nadsonia* | Spherical | Warty | 1 |
| *Monosporella* | Needle-shaped | | 1 |
| *Nematospora* | Spindle-shaped (two bundles per ascus) | Flagellum at one end (immotile) | 8 |
| *Coccidiascus* | Fusiform | ................ | 8 |
| *Lipomyces* | Oval | Smooth | 4–16 |

**Methods of Inducing Sporulation.** There are a number of methods for inducing sporulation, some of which have been reviewed by Henrici (1941), Lindegren and Lindegren (1944, 1950), Phaff and Mrak (1948, 1949), Prescott and Dunn (1949), and Tremaine and Miller (1956). They involve the use of vegetables and/or fruit, nonnutrient, or other substrates. Failure of one method to induce sporulation in an unknown species does not necessarily mean that the yeast is asporogenous. A number of different methods may have to be used before one arrives at this conclusion.

Substrates used for inducing sporulation include the stock medium of Mrak, Phaff, and Douglas (1942); the presporulation medium of Lindegren and Lindegren (1944); the vegetable-juice medium of Henrici (1941), as modified by Wickerham, Flickinger, and Burton (1946); the carrot-infusion agar (McKelvey); Gorodkawa's medium; Kufferath's medium; the soil-extract–gelatine medium; carrot or potato plugs; cucumber wedges; grape or prune juice; raisin agar; sterilized sausages (Stelling-Dekker); sugar solutions; the acetate-dextrose medium of Adams (1949); 0.1 per cent sodium acetate in distilled water (Tremaine and Miller, 1956); and plaster of Paris blocks or slants.

**Basic Research concerning Sporulation.** Considerable research relating to sporulation has been carried out by Miller and associates, some of which is referred to below.

Adams and Miller (1954) observed that ascospore formation by *S. cerevisiae,* in the absence of carbon dioxide, was stimulated by oxygen in concentrations up to 12 per cent, was slightly depressed by a concentration of 41.1 per cent, and was depressed to greater extents by concentrations of 68.8 and 100 per cent. Carbon dioxide inhibited ascospore formation, and very few ascospores were found at carbon dioxide concentrations of 25 per cent or higher. The vapors from a 5 per cent solution of ethanol markedly inhibited sporulation.

Miller and Halpern (1956) found that optimum sporulation of bakers' yeast occurred in 0.033 to 0.1 per cent glucose, 1 per cent pyruvate, 0.033 per cent ethanol, 0.033 per cent aldehyde, or 0.033 per cent acetate. Sporulation was suppressed by 0.33 per cent glucose, 1 per cent ethanol, 0.1 per cent aldehyde, or 0.3 per cent acetate.

Tremaine and Miller (1954) studied the effects of six vitamins on ascospore formation by bakers' yeast. They discovered that the vitamin content of the presporulation medium had a large influence on the yields of asci in the sporulation medium (0.1 per cent sodium acetate). Unless biotin was present in the presporulation medium, the yields of asci were low. The addition of niacin, thiamin, pyridoxine hydrochloride, and inositol to the presporulation medium, along with biotin and calcium pantothenate, stimulated sporulation.

Miller (1957) found maximum yields of asci of bakers' yeast in 0.05 per cent concentrations of glucose, fructose, and mannose. The yields declined markedly as the concentrations of these sugars were increased. The maximum yields of three- and four-spored asci were observed at 0.1 per cent.

**Yeast Genetics.** The nucleus of the yeast cell is concerned with heredity. Each nucleus contains one or two sets of "chromosomes," the bearers of heredity. The number of chromosomes in a basic set is usually constant for a species. For example, the fruit fly (*Drosophila melanogaster*) contains 4 chromosomes in its basic set, while man (*Homo sapiens*) contains 24 chromosomes in the basic set. Each chromosome in turn contains "genes," the determiners of heredity.

A reproductive cell is known as a "gamete," which in the higher forms of life is usually designated as an egg or sperm. However, in the lower forms of life, where no simple method is available for differentiating between the cells, they may be referred to as male and female or as plus or minus cells. Each gamete carries one set of chromosomes in its nucleus. The basic number of chromosomes for a species is known as the "haploid" or "monoploid" number. This may be designated as the $1n$ or $1x$ number. Twice the basic number of chromosomes, such as is found in a zygote resulting from the fusion of two gametes, is known as the "diploid" number and may be designated as the $2n$ or $2x$ number.

When two gametes of the same sex (or mating types of the same kind) fuse, there results a homozygous diploid, which is known as an "illegitimate" diploid. Thus, the union of two plus or two minus gametes of haploid nature gives rise to an illegitimate diploid. On the other hand, the fusion of a plus gamete with a minus gamete gives rise to a heterozygous diploid, known as a "legitimate" diploid.

In the case of *S. cerevisiae*, there are believed to be two mating types, $a$ and $\alpha$. If two gametes of mating type $a$ or two gametes of mating type $\alpha$ fuse, the zygote will contain the diploid number of chromosomes $aa$ or $\alpha\alpha$. However, if the $a$ type fuses with the $\alpha$ type, the resultant cell will be a legitimate diploid $a/\alpha$ (refer to Fig. 4).

According to Lindegren, most bakers' and brewers' yeasts (strains of *S. cerevisiae*) are illegitimately diploid. Illegitimate diploids usually form fewer viable ascospores. These homozygous illegitimate diploids are fairly stable, more so than the heterozygous legitimate diploids, because they do not usually form ascospores and because they are homozygous. The legitimate diploids are stable as long as they reproduce vegetatively (by budding). However, sporulation may lead to a heterogamous mixture. Legitimate diploids produce four ascospores and occur commonly in nature.

In the case of *S. cerevisiae*, haploid cells differ from diploid cells in several respects. In general, the haploid cells are smaller, rounder, and rather variable. Such cells do not usually sporulate, and when they do, the spores usually degenerate. The cells tend to grow in clusters. Colonies from such cells are usually rough, small, and variable. Diploid cells are generally larger and give rise to relatively large, smooth, and uniform colonies (Lindegren, 1945).

A zygote may be formed from the fusion of two ascospores (endospores), of an ascospore and a vegetative haploid cell, or two vegetative haploid cells, of two sister haploid cells, or mother and daughter haploid cells, etc.

According to Lindegren, yeasts may be classified into two groups on the basis of their sexual behavior. In the first group, he places the genera *Schizosaccharomyces*, *Zygosaccharomyces*, *Zygopichia*, *Debaryomyces*, *Nadsonia*, and *Nematospora*. The vegetative cells of species of these genera are haploid. Following nuclear fusion, the diploid stage exists for a brief time but is followed by meiosis. The ascospores are haploid and give rise to haploid cells. In the second group, Lindegren places the genera *Saccharomyces*, *Saccharomycodes*, and *Hansenula*. Here the vegegetative cells are diploid and the ascospores are haploid. Meiosis takes place during sporulation. Haploid vegetative cells, usually small and

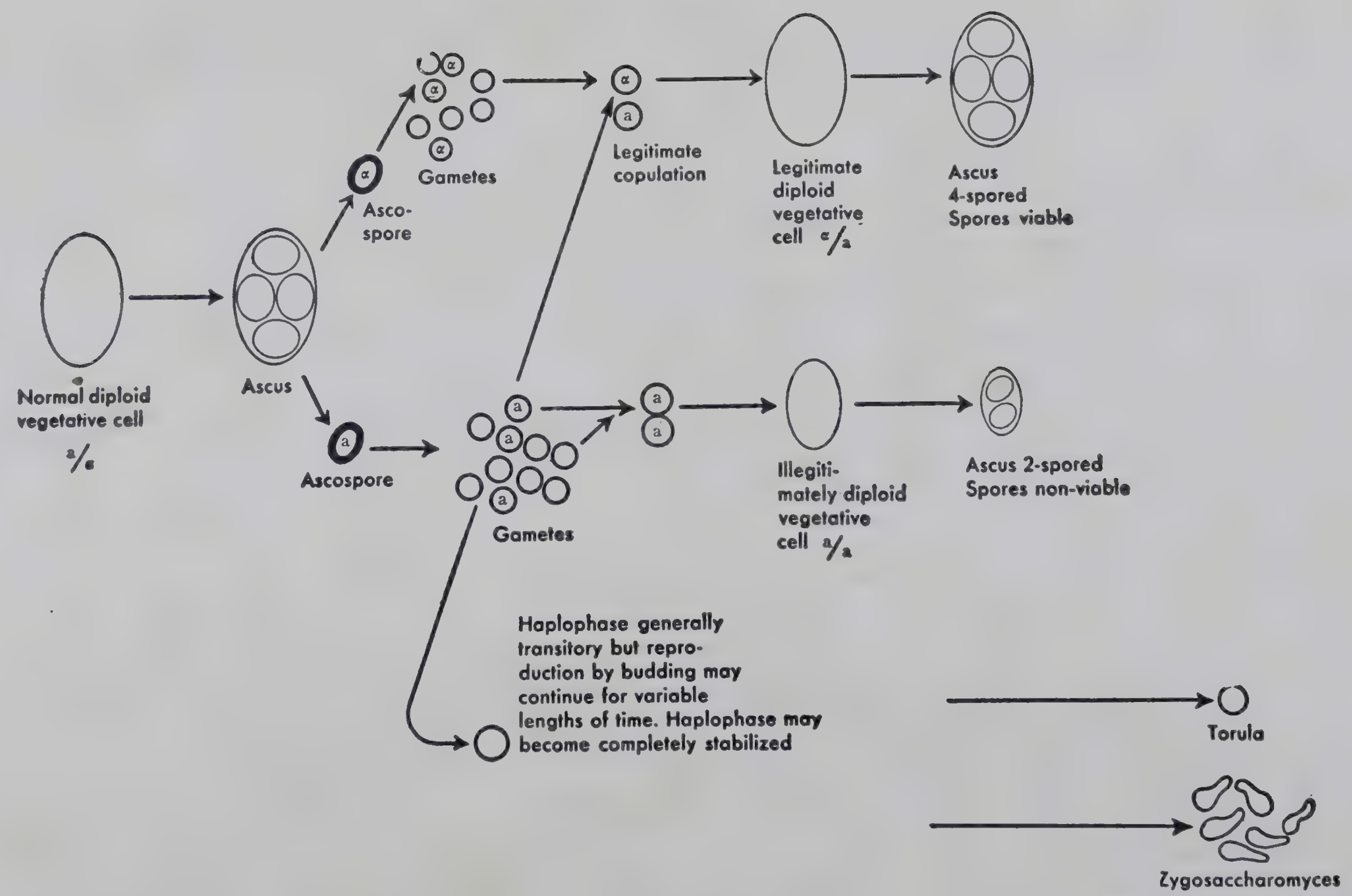

Normal diploid vegetative cell
a/α
Ascus
Asco-spore
α
Ascospore
a
Gametes
α
a
Legitimate copulation
Legitimate diploid vegetative cell α/a
Ascus 4-spored Spores viable
Illegitimately diploid vegetative cell a/a
Ascus 2-spored Spores non-viable
Haplophase generally transitory but reproduction by budding may continue for variable lengths of time. Haplophase may become completely stabilized
Torula
Zygosaccharomyces

FIG. 4. Life cycle of *Saccharomyces cerevisiae*, showing the relationship with *Torula* and *Zygosaccharomyces*. The ascospores belong to two mating types *a* and *α*, and the heterozygote is the legitimate diploid. The homozygotes are called "illegitimate diploids." [*Courtesy of C. C. Lindegren, Wallerstein Labs. Communs.*, 7(22): 153 (1944).]

lacking vigor, may arise from haploid spores. These spores or vegetative cells formed from them may fuse and produce diploid cells, which are vigorous.

The breeding of yeasts has large possibilities. Winge and Laustsen produced a new hybrid by breeding together bakers' yeast and *S. validus*. The resultant hybrid was an improvement over the standard bakers' yeast. A hybrid produced by mating haplophase cultures of *S. cerevisiae* with haplophase cultures of *S. carlsbergensis* was able to synthesize biotin, pantothenic acid, and pyridoxine, vitamins of the B complex, in large amounts, according to Lindegren and Lindegren (1945). *S. cerevisiae* was unable to synthesize either biotin or pantothenic acid but able to synthesize pyridoxine. *S. carlsbergensis* was able to synthesize both biotin and pantothenic acid in large amounts but unable to synthesize pyridoxine.

The subject of yeast genetics is one of large and growing importance. The interested reader is referred to the books "The Yeast Cell, Its Genetics and Cytology," by Lindegren; "The Biology of the Yeasts," by Ingram; "The Chemistry and Biology of Yeasts," by Cook and others; as well as to the publications of the Lindegrens and their associates; Winge, Laustsen, and coworkers; Wickerham and Burton; and Fowell and others, some of which are cited at the end of this chapter.

**Chemical Composition.** Yeasts contain 68 to 83 per cent of moisture. The yeast cell is composed of nitrogen-containing compounds, carbohydrates, lipids, vitamins, minerals, and other substances.

Nitrogen-containing compounds are shown in Table 8. The usual nitrogen content of yeast varies from 7 to 9 per cent but may be as low as 2.5 per cent or as high as 14 per cent. A large percentage of the total nitrogen (about 64 to 76 per cent) is found in the pure proteins, about 10 per cent of it occurs in the purine bases, and approximately 4 per cent

TABLE 8. NITROGEN-CONTAINING COMPOUNDS IN YEAST *

| Principal compounds | Minor compounds | |
|---|---|---|
| Proteins | Adenosine triphosphate | Vitamins |
| Amino acids | Adenylic acid | Biotin |
| Purines | Cephalin | Folic acid |
| Adenine | Coenzymes | Nicotinic acid |
| Guanine | Cocarboxylase | Para-aminobenzoic acid |
| Pyrimidines | Coenzyme I † | Pyridoxine |
| Cytosine | Coenzyme II ‡ | Riboflavin |
| Uracil | Cytochrome | Thiamin |
| | Glucosamine | |
| | Glutathione | |
| | Lecithin | |

* C. G. Dunn, *Wallerstein Labs. Communs.*, **15**(48) : 61 (1952).

† Coenzyme I is diphosphopyridine nucleotide (DPN).

‡ Coenzyme II is triphosphopyridine nucleotide (TPN).

of it occurs in the pyrimidines. About 15 per cent of the total nitrogen occurs in the amino acids, nucleotides, and other products.

The amino acid composition of some yeasts is shown in Table 9.

TABLE 9. AMINO ACID COMPOSITION OF SOME YEASTS [a]

(Percentages of dry matter)

| | Brewers' yeast [b] | Yeast [c] | Animal feed yeast [d] | *Saccharomyces cerevisiae* [e] | *Torula* [f] | *Hansenula* | *Rhodotorula rubra* |
|---|---|---|---|---|---|---|---|
| Alanine | 2.7 | ... | ... | ... | ... | ... | ... |
| Arginine | 1.5 | 4.3 | 2.0 | 2.4 | 3.1 | 2.9 | 3.7 |
| Cystine | [g] | 1.0 | ... | ... | ... | ... | ... |
| Glutamic acid | ... | ... | 5.4 | ... | ... | ... | ... |
| Histidine | 1.5 | 2.8 | 1.4 | 2.7 | 1.5 | 1.4 | 1.99 |
| Isoleucine | 2.8 | 5.9 | 3.4 | 2.5 | 3.7 | 3.7 | 2.1 |
| Leucine | 3.4 | 7.4 | 3.4 | 3.8 | 3.8 | 3.6 | 3.3 |
| Lysine | 3.2 | 7.5 | 3.3 | 3.1 | 4.4 | 4.3 | 3.0 |
| Methionine | [g] | 2.7 | 1.5 | 0.65 | ... | ... | 0.53 |
| Phenylalanine | 2.1 | 4.1 | 2.6 | 2.1 | 2.3 | 2.4 | 1.72 |
| Serine | ... | ... | 4.2 | ... | ... | ... | ... |
| Threonine | 2.6 | 5.5 | 2.4 | 2.4 | 2.5 | 2.4 | 1.79 |
| Tryptophan | 0.6 | 1.3 | 0.6 | 0.59 | 0.3 | 0.3 | 0.45 |
| Tyrosine | ... | 3.6 | 1.0 | ... | ... | ... | ... |
| Valine | ... | 5.0 | 2.9 | 2.8 | 3.3 | 3.3 | 2.5 |

[a] Courtesy of C. G. Dunn, *Wallerstein Labs. Communs.*, **15**(48): 61 (1952).

[b] Roth and Zander (1944).

[c] Block and Bolling (average values) (1945).

[d] Baumgarten, Mather, and Stone (1946).

[e] Stokes and Gunness (1946).

[f] Kurth and Cheldelin (1946).

[g] Combined value = 1.7.

Neuberg (1946) has reviewed the data concerning the carbohydrate constituents of yeasts. The chief ones are glycogen, hexosephosphates, desoxyribose, trehalose, amylose, gum, dulcitol, and cellulose.

The lipids of yeasts consist of the glycerides of fatty acids, phospholipids, and sterols. The glycerides of hexadecanoic, linoleic, myristic, oleic, palmitic, stearic, and other fatty acids are found in the fats of yeasts. Lecithin and cephalin, which are usually available in a ratio of 3:1, are the phospholipids. Ergosterol is the most important sterol, but small quantities of zymosterol and cervisterol may be found.

The food yeasts are regarded as excellent sources of vitamins of the B complex. Data concerning the vitamin contents of some of them are presented in Table 10.

Chiao and Peterson (1953) found the methionine content of seven commercial yeasts to vary from 0.48 to 0.75 per cent on a dry basis, while *Rhodotorula glutinis* (*gracilis*), a fat yeast, contained 1 per cent.

Phosphorus, potassium, calcium, and other important elements are found in the ash of yeast, as is indicated in Tables 11, 14, and 15.

## TABLE 10. THE VITAMIN CONTENTS OF SOME FOOD YEASTS *

(Micrograms per gram of dried yeast)

| Food yeast | Thiamin | Riboflavin | Nicotinic acid | Panto-thenic acid | Pyri-doxine | Folic acid | Biotin | *p*-Amino-benzoic acid | Medium | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| Bakers' compressed | 20–40 | 60–85 | 200–700 | 180–330 | ..... | ....... | 0.6–1.8 | ..... | .............. | Massock and Baldwin (1943). |
| Bakers' foil | 80–150 | 50–65 | 180–400 | 120–160 | ..... | ....... | 0.5–1.5 | ..... | .............. | Massock and Baldwin (1943). |
| Brewers' | 104–250 | 25–80 | 300–627 | 72–86 | 23–40 | 19–30 | 1.1 | 15–40 | .............. | Peterson (1948). |
| *Candida arborea* | 31.3–33.1 | 52.3–69.5 | 492.3–580.2 | ....... | ..... | 14.8–16.0 | ....... | ..... | Molasses, beet and cane | Agarwal et al. (1947). |
| ***C. arborea*** | **13–33** | **46–70** | **301–580** | ....... | ..... | **12–26** | **0.24–3.2** | **11–21** | .............. | **Peterson (1948).** |
| *Hansenula* strain | 8.5 | 54 | 590 | 180 | ..... | 1.7 | 1.7 | 16 | Wood sugar stillage | Kurth and Cheldelin (1946). |
| *Mycotorula* strain | 5.3 | 59 | 600 | ....... | ..... | 3.1 | 1.8 | 31 | Wood sugar stillage | Kurth and Cheldelin (1946). |
| ***Oidium lactis*** | **12–29** | **40–55** | **186–248** | | ..... | **6–15** | **1.3–2.1** | ..... | .............. | **Peterson (1948).** |
| *O. lactis* | 20.1–29.0 | 39.9–55.0 | 192.8–247.5 | ....... | ..... | 5.6–7.8 | ....... | ..... | Molasses, beet and cane | Agarwal et al. (1947). |
| *Saccharomyces cerevisiae* | 28–41 | 39–62 | 277–568 | ....... | ..... | 19–36 | 0.45–3.6 | 11–62 | .............. | Peterson (1948). |
| *Torulopsis utilis* † (Wisconsin pulp mill, commercial) | 5.3 | 42.7 | 417.3 | 39.2 | 33.4 | 21.5 | 2.3 | ..... | Spent sulfite liquor | Wiley et al. (1950). |
| *T. utilis* | 35.4–38.1 | 54.2–62.0 | 511.3–600.0 | ....... | ..... | 10.6–15.2 | ....... | ..... | Molasses, beet and cane | Agarwal et al. (1947). |
| ***T. utilis*** | **22** | **54** | **440–490** | ....... | ..... | ........ | ....... | ..... | **Molasses, beet and cane** | **Colonial Food Yeast, Inc. (1944).** |

---

* C. G. Dunn, *Wallerstein Labs. Communs.*, **15**(48): 61 (1952).

† Average analysis.

Table 11. Some Ash Constituents of Yeast *

| | Per Cent |
|---|---|
| Phosphorus pentoxide | 54.5 |
| Potassium oxide | 36.5 |
| Magnesium oxide | 5.2 |
| Calcium oxide | 1.4 |
| Silicon oxide | 1.2 |
| Sodium oxide | 0.7 |
| Sulfur trioxide | 0.5 |
| Chlorine | traces |
| Iron | traces |

* C. N. Frey, *Ind. Eng. Chem.*, 22: 1154 (1930). Copyright 1930 by the Americ Chemical Society and reprinted by permission of the copyright owner.

**Pigments.** A number of pigments have been isolated from yeast particularly from species of *Rhodotorula* and *Cryptococcus,* two asporogenous genera. Modern methods have demonstrated the presence o pigments in a comparatively large number of yeast species.

Nakayama, Mackinney, and Phaff (1954) found that the "yello yeasts" of the genera *Rhodotorula* and *Cryptococcus* contained beta carotene as the chief pigment and that its content varied from very lo amounts to 10 mg. per 100 g. dry yeast in certain species of *Cryptococcu* The principal pigments of *R. rubra* were torularhodin, torulin, β-caroten and gamma-carotene.

Carotenoid pigments are used as a basis for separating the genus *Rhodotorula* from the genera *Cryptococcus* and *Torulopsis* by Lodder an Kreger-van Rij (1952). However, in view of the fact that these pigment appear in a greater number of species than originally suspected, th taxonomic value of carotenoid pigment production has been questione

Pulcherrimin, which contains a high iron content, is a red, noncarotenoid pigment formed by red mutants of *Candida pulcherrima* and othe yeast species. Iron and oxygen are essential for the formation of this pigment. Biotin deficiency is a factor of importance also, in the formation o this pigment. *C. pulcherrima* and its pigment have been studied extensively by Van der Walt (1952).

Methods used for demonstrating the presence of yeast pigments hav been described by Van der Walt (1952), Nakayama et al. (1954), Peterson and associates (1954), and others. The last-named researchers foun that the carotenoid pigments of the red- and yellow-pigmented types o species of *Rhodotorula* could be extracted directly from the yeast cel with cold acetone after growth in Wickerham's synthetic broth (containing 2 per cent glucose) for 72 hr. on a rotary shaker. The pigments wer transferred from the acetone to petroleum ether and characterized b chromatographic and spectrophotometric methods. Pigments of certai species of yeasts from the *R. flava* group were altered or destroyed b preliminary treatment of the cells with hydrochloric acid and heat.

**Methods of Isolating Pure Cultures of Yeast.** In order to make su that the culture isolated is pure and that it is not in reality a mixe culture of two morphologically related varieties of yeast or an undesirable combination of microorganisms, it is necessary to isolate single cells by special technique and to observe these cells during reproduction. Although there are several methods by which pure cultures may be isolated, the methods cited here will be restricted to the more important ones. Skill and patience are required in some of the methods of single-cell isolation.

1. *Moist-chamber Method of Hansen.*[1] The chamber (Fig. 5) consists of a glass slide, a glass ring, and a cover slip with numbered squares (usually 16). The cover slip is attached to the ring by glass cement, wax, or a petroleum-jelly–wax mixture. Before the chamber is used, the component parts should be sterilized by being carefully flamed.

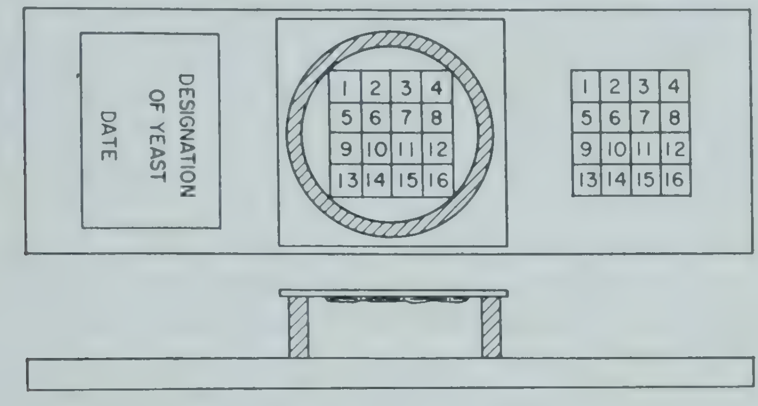


FIG. 5. Moist chamber for Hansen method.

The culture of yeast from which the single-cell isolation is desired is diluted with sterile beer wort or water until a drop contains only a few cells. A drop of the diluted culture is then placed in a tube of sterile melted wort gelatin, and the tube is thoroughly agitated to distribute the yeast cells uniformly throughout the medium. One drop of this medium is spread out on a glass slide, and, by means of the 100× magnification of a microscope, the slide is examined for the approximate number of cells. If the number is satisfactory (20 or less), a small drop of the liquid wort gelatin containing the cells is placed on the underside of the cover slip and spread thinly and uniformly over the numbered squares. The chamber is placed so that the gelatin rests in an even layer on the cover slip until it has solidified. Afterward the chamber is inverted, a drop of sterile water is placed on the slide to ensure a moist atmosphere in the chamber, and the ring is sealed tightly to the slide by means of petroleum jelly. The single cells are then located microscopically, and their positions are mapped on a diagram corresponding to the marked cover slip. Several moist chambers are usually prepared at the same time. They are then incubated at room temperature or around 25°C. for 2 or more days, during which time the growing yeast cells are carefully observed. During this time the single cells will have developed into colonies. Tubes of sterile wort are then inoculated from the individual colonies known have grown from single cells.

---

[1] A. Jorgensen, "Practical Management of Pure Yeast," revised by A. Hansen. Charles Griffin & Co., Ltd., London, 1936.

2. *The Lindner Method.* This method is a modification of the Hans procedure. The culture containing the desired yeast is diluted in ster wort, cider, or grape juice. By means of a sterile crow-quill pen or wi five rows of droplets, five droplets to a row, are deposited on a ster cover slip that may or may not be marked into numbered squares. T cover slip is placed, culture side down, over the concavity of a steri hollow ground-glass slide or ring slide after a drop of sterile water placed in the chamber. It is sealed with petroleum jelly or paraffin. T droplets are examined microscopically, and those containing a single c are marked by drawing small circles around them, or, if the squares a numbered, the locations are recorded by sketching the position of t droplet on a corresponding drawing. After incubation and observatio the cultures are transferred to sterile wort.

3. *Dilution-plate Method.* Tubes of dextrose, malt, or other suitab agar are melted and cooled to 42 to 44°C. By means of a loop, a tube agar is inoculated with the yeast-containing culture. The tube is tho oughly shaken, and a unit amount of this medium is transferred to second tube of melted agar. A third tube is inoculated in the same mann from the second tube. The contents of each of the three tubes are poure into petri dishes, and the plates are incubated at 25 to 30°C. for 2 or mo days. When the colonies have developed, samples of those which appe to consist of pure yeasts are examined with the microscope, using hangi drops. If the yeast appears to be pure and satisfactory, sterile wort ma be inoculated from the colony. This method, though simple, does n ensure the isolation of a culture from a single cell.

4. *The Micromanipulator Method.* The micromanipulator may used successfully in isolating pure cultures of yeasts from single cel In experienced hands, this method is rapid. The euscope, attached the microscope, aids in the isolation of single cells.

5. *Other Methods.* Techniques similar to those used in bacteriolog such as streaking, may also be employed for isolating pure cultures yeasts.

**Media Used for Isolating Yeasts.** A number of different media ma be used for the isolation of yeasts. These must contain the nutrien required for the satisfactory growth of yeasts but in addition may conta ingredients added to inhibit the growth of bacteria and molds. Wicke ham (1951) used a yeast extract–malt extract medium of the followi composition (per liter) to isolate yeasts: 3 g. malt extract, 3 g. yea extract, 5 g. peptone, 10 g. glucose, and 20 g. agar. The pH was lower to 3 to 4 in order to isolate yeasts from samples containing a high pe centage of bacteria. Sterile paraffin oil was added in a layer 1 cm. de to prevent mold growth. Carson et al. (1956) used malt extract agar a pH of 4.5 to isolate yeasts from the natural breeding sites (slime flux etc.) of *Drosophila*. Miller and Webb (1954) incorporated 0.003 per ce rose bengal, 1 per cent ox gall, or lactic acid to produce a pH of about into potato dextrose agar in order to detect and isolate yeasts from so

All three additives inhibited the development of bacterial colonies; whereas rose bengal and ox gall reduced the interference of spreading fungi, lactic acid did not. Phaff et al. (1952) used potato dextrose agar at a pH of 3.5 to isolate yeasts from shrimp. Miller and Mrak (1953) used a wort propionate agar for the isolation of yeasts from dried-fruit beetles in figs. The wort (5° Brix) contained 0.25 per cent sodium propionate and was quite effective in inhibiting molds. Etchells et al. (1954) found that acidified dextrose agar containing 0.35 per cent sodium propionate was the most effective medium tested for separating yeasts from molds in samples of soil. However, the propionate completely inhibited *R. glutinis* and strongly inhibited *Endomycopsis ohmeri* in low concentrations and resulted in low yeast counts, in comparison with acidified dextrose agar and synthetic agar, in plant-material samples. The yeast nitrogen base broth (Difco) of Wickerham, modified by the addition of 2 per cent agar, 2 per cent dextrose, and acidification (referred to as "acidified synthetic agar"), was the medium of choice for the enumeration and isolation of yeasts from plant material.

**The Isolation of a Single Spore.** Winge and Laustsen (1937) regard the one-spore culture as being the smallest biological unit in yeasts instead of the single cell.

Laustsen has developed a technique for isolating all the spores of an ascus and cultivating them. His method is as follows: The yeast is caused to sporulate by being placed on a plaster block and incubated for at least 30 hr. at room temperature. By means of aseptic technique, a small drop of wort on a cover slip is inoculated with the spore material. The cover slip is then placed on a special operating chamber (Fig. 6).

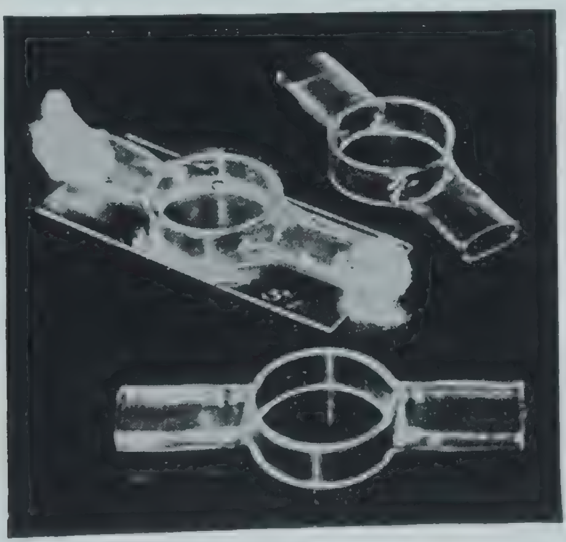


FIG. 6. Chamber used in isolating single spores. (*Courtesy of Ö. Winge, Carlsberg Laboratory, Copenhagen.*)

An ascus is selected and withdrawn from the droplet with a very fine glass needle. A small amount of wort naturally adheres to the ascus—more may be readily added if desired.

The isolation (Fig. 7) is carried out by means of two special gla needles. One needle, which has a point about 7 microns in diameter, introduced through one opening of the operating chamber (Fig. 6); th other needle, which has a point approximately 2.5 microns in diamete is introduced through the other opening. The two needles are manipu lated in such a manner as to burst the wall of the ascus against the surfac

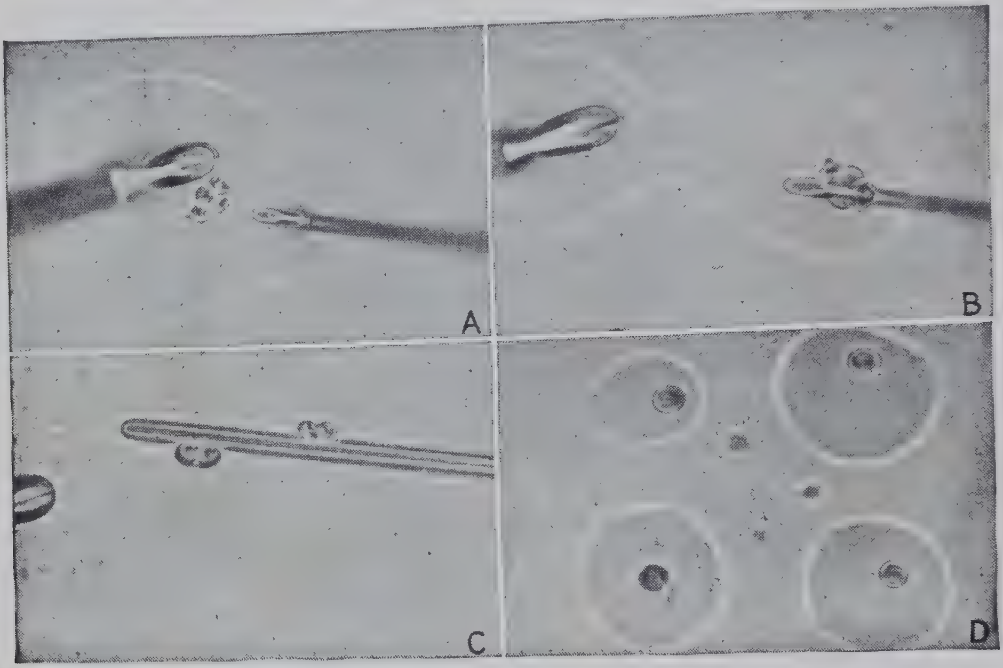


Fig. 7. The isolation of single spores. (*Courtesy of Ö. Winge, Carlsberg Laborator* *Copenhagen.*)

of the cover slip. Each of the spores liberated is pulled into a separat droplet of sterile wort, which has been placed previously on the cover sli

After the spore has germinated and a colony of cells has develope some of the cells are transferred by the use of a sterile platinum need to a Freudenreich flask containing sterile wort. Some of the remainin cells may be transferred to a second flask by using a sterile, pointed pie of filter paper.

**Identification of Yeasts.** In order to identify an isolate it is necessa to study certain morphological and physiological or biochemical chara teristics. Data relating to the shape and size of the cells; myceliu pseudomycelium, budding cells, arthrospores; the manner of vegetati reproduction—by budding and/or fission; the formation of asci, as relat to conjugation; the shape and number of ascospores; and the formation ballistospores, chlamydospores, or blastospores are some of the morph logical characteristics studied. Giant colonies, pellicle formation, a other growth characteristics may also furnish helpful information.

Physiological tests include those concerned with carbon and nitrog assimilation, fermentation, vitamin-deficiency patterns, and ability grow at different osmotic pressures, to liquefy gelatin, to produce star and to form esters. Lodder and Kreger-van Rij (1952) used the suga glucose, galactose, sucrose, maltose, and lactose, and ethyl alcohol carbon sources. In the identification of *Hansenula* species Wickerham (1951) included, in addition, trehalose, L-sorbose, cellobiose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine hydrochloride, glycerol, *i*-erythritol, adonitol, dulcitol, D-mannitol, D-sorbitol, alpha-methyl-D-glucoside, salicin, potassium D-gluconate, calcium 2-keto-D-gluconate, potassium 5-keto-D-gluconate, potassium sodium saccharate, pyruvic acid, DL-lactic acid, succinic acid, citric acid, ethyl acetoacetate, and *i*-inositol. Potassium nitrate is used as a nitrogen source, since there are considerable numbers of species which do not utilize it. Glucose, galactose, sucrose, maltose, lactose, and raffinose are the usual sugars employed to indicate fermentation, or gas production, by yeasts. Since the requirement for vitamins varies, a study of vitamin-deficiency patterns may be helpful in separating strains, as in *S. cerevisiae* (Schultz and Atkins, 1947).

For an intensive review of this subject, the reader is referred to the works of Lodder and Kreger-van Rij, Wickerham, Mrak and Phaff, and others.

**Classification of Yeasts.** The problem of classification of the yeasts is one of great difficulty and requires the concerted efforts of specialists for solution. Rewarding advances have been made in this field by Mrak and Phaff (1949), Wickerham (1951, 1952), Lodder and Kreger-van Rij (1952), Phaff (1956), Cook (1958), and others.

A number of publications should prove particularly helpful in the classification of yeast isolates. The book "The Yeasts, a Taxonomic Study," by Lodder and Kreger-van Rij, represents a comprehensive coverage of the genera and species found in the families Saccharomycetaceae, Sporobolomycetaceae, and Cryptococcaceae. It contains fewer species and varieties than earlier classifications and provides for easier identification of isolates. For the classification of species of the genus *Hansenula,* refer to the publication of Wickerham on this subject.

A description of the subdivisions of the family Saccharomycetaceae follows, while a classification of the asporogenous yeasts is presented in Table 12.

Lodder and Kreger-van Rij [1] (1952) describe the subdivisions of the *Saccharomycetaceae* as follows:

I. Subfamily *Eramascoideae*

Mycelium only, vegetative reproduction by fission, ascus formation after preceding conjugation, spores up to 8 per ascus, dissimilation oxidative.

Genus 1. *Eremascus* Eidam

II. Subfamily *Endomycetoideae*

Mycelium and/or arthrospores; vegetative reproduction by fission; ascus formation usually after preceding conjugation; spores round, oval, hat-shaped; dissimilation oxidative or fermentative.

Genus 1. *Endomyces* Reess
Gametangia present

---

[1] Quoted by permission from J. Lodder and N. J. W. Kreger-van Rij, "The Yeasts, a Taxonomic Study," North Holland Publishing Company, Amsterdam, 1952.

## Table 12. Classification of the Asporogenous Yeasts *

Family Cryptococcaceae

Budding cells, moreover pseudomycelium, mycelium, and arthrospores may be form
Cells hyaline, never dark or brown, carotenoid pigments may be formed.
Dissimilation strictly oxidative or also fermentative.

| Subfamily Cryptococcoideae | Subfamily Trichosporoideae | Subfamily Rhodotoruloideae |
|---|---|---|
| Budding cells, occasionally pseudomycelium and mycelium, no arthrospores.<br>No yellow to red color (no carotenoid pigments). | Budding cells, pseudomycelium, mycelium, and arthrospores.<br>No yellow to red color (no carotenoid pigments).<br>Occasionally fermentation. | Budding cells, occasiona pseudomycelium, no a throspores.<br>Yellow to red color (carotenoid pigments)<br>No fermentation. |
| Genera | Genus | Genus |
| 1. *Cryptococcus*<br>Multilateral budding, no pseudomycelium or mycelium.<br>Capsulated cells. Formation of starch-like compounds.<br>No fermentation. | 1. *Trichosporon* | 1. *Rhodotorula* |
| 2. *Torulopsis*<br>Multilateral budding, no pseudomycelium or mycelium.<br>No formation of starch-like compounds.<br>Mostly fermentation. | | |
| 3. *Pityrosporum*<br>Polar budding.<br>Oval or bottle-shaped cells.<br>No fermentation. | | |
| 4. *Brettanomyces*<br>Multilateral budding.<br>Round, oval or "ogive-shaped" cells.<br>Slow growth.<br>Strong acid production.<br>Fermentation. | | |
| 5. *Candida*<br>Multilateral budding, pseudomycelium, occasionally mycelium.<br>Fermentation or no fermentation. | | |
| 6. *Kloeckera*<br>Bipolar budding.<br>Lemon-shaped cells.<br>Fermentation. | | |
| 7. *Trigonopsis*<br>Multilateral or triangular budding.<br>Oval or triangular cells.<br>No fermentation. | | |

* Courtesy of J. Lodder and N. J. W. Kreger-van Rij, *Lab. Prac.*, **3**(12): 4 (1954).

Genus 2. *Schizosaccharomyces* Lindner
Gametangia absent, conjugation somatogamous

III. Subfamily *Saccharomycetoideae*

Mycelium and budding cells, pseudomycelium and/or loose budding cells; vegetative reproduction by fission and budding, or budding only; conjugation may or may not immediately precede ascus formation; spores of various shapes, 1 to 4 per ascus; dissimilation from merely oxidative to predominantly fermentative.

Tribe *Endomycopseae*

Mycelium and budding cells; vegetative reproduction by fission and by multilateral budding; dissimilation mainly oxidative but also fermentative.

Genus 1. *Endomycopsis* Dekker

Tribe *Saccharomyceteae*

No mycelium; pseudomycelium and/or loose budding cells; vegetative reproduction by multilateral budding; dissimilation from merely oxidative to predominantly fermentative.

Genus 2. *Saccharomyces* (Meyen) Reess
Pseudomycelium and/or loose budding cells varying in shape from round to elongate; conjugation may or may not immediately precede ascus formation; spores usually round or oval, also reniform or hat-shaped, 1 to 4 per ascus; spores may conjugate; dissimilation besides oxidative always fermentative; nitrate not assimilated.

Genus 3. *Pichia* Hansen
Pseudomycelium and loose budding cells which are mainly oval or long-cylindrical; conjugation may or may not immediately precede ascus formation; spores hemispherical, hat-shaped, angular or round, 1 to 4 per ascus; dissimilation principally oxidative—pellicle formation—to a lesser extent fermentative; nitrate not assimilated.

Genus 4. *Hansenula* H. et P. Sydow
Pseudomycelium and loose budding cells varying in shape from round to elongate; conjugation may or may not immediately precede ascus formation; spores hat-shaped, Saturn-shaped or round, 1 to 4 per ascus; dissimilation oxidative—pellicle formation—besides usually vigorously fermentative; nitrate assimilated.

Genus 5. *Schwanniomyces* Klocker
Oval cells; conjugation does not immediately precede ascus formation; protuberances resembling conjugation tubes may be formed; spores round, warty, with a ledge, 1 or seldom 2 per ascus; dissimilation mainly fermentative; nitrate not assimilated.

Genus 6. *Debaryomyces* Lodder et v. Rij nom. conserv.
Mainly round to oval cells, rarely pseudomycelium; usually heterogamous conjugation immediately precedes ascus formation; spores round, often with more or less distinct warts, 1 or seldom 2, exceptionally 4 per ascus; dissimilation almost entirely oxidative; nitrate not assimilated.

Tribe *Nadsonieae*

No mycelium, budding cells and occasionally pseudomycelium; vegetative reproduction by bipolar budding on a more or less broad base; dissimilation oxidative and fermentative.

Genus 7. *Saccharomycodes* Hansen
Cells usually lemon-shaped; no cell conjugation before ascus formation 4 spores per ascus which conjugate in pairs; spores round; dissimilatio besides oxidative also fermentative.

Genus 8. *Hanseniaspora* Zikes
Cells usually lemon-shaped; no conjugation observed; 1 to 4 spores pe ascus, first round, later hat-shaped; dissimilation besides oxidative also fermentative.

Genus 9. *Nadsonia* Sydow
Cells usually lemon-shaped; heterogamous conjugation between mothe cell and bud after which a bud of the zygote is transformed into an ascus 1, seldom 2, spores per ascus which are round, warty, light brown; dissimilation oxidative—pellicle formation—besides fermentative.

IV. Subfamily *Nematosporoideae*

Mycelium and/or budding cells; vegetative reproduction by fission and/o multilateral budding; conjugation may precede ascus formation; spores fusiform or needle-shaped with or without a whip; dissimilation fermentative beside oxidative.

Genus 1. *Monosporella* Keilin
No mycelium; cells oval; no conjugation; spores needle-shaped withou whip, only 1 per ascus.

Genus 2. *Nematospora* Peglion
Mycelium and budding cells; no conjugation preceding ascus formation spores fusiform with a whip, up to 8 per ascus; dissimilation besides oxidative also fermentative.

Genus 3. *Coccidiascus* Chatton
Cells round or oblong, no mycelium; isogamous conjugation precedes ascu formation; 8 fusiform spores—without whips—per ascus.

V. Subfamily *Lipomycetoideae*

Budding cells only; sac-like ascus formed by vegetative cells as a protuberance of the latter; spores oval, light reddish, up to 16 per ascus; dissimilatio strictly oxidative.

Genus 1. *Lipomyces* nov. gen.

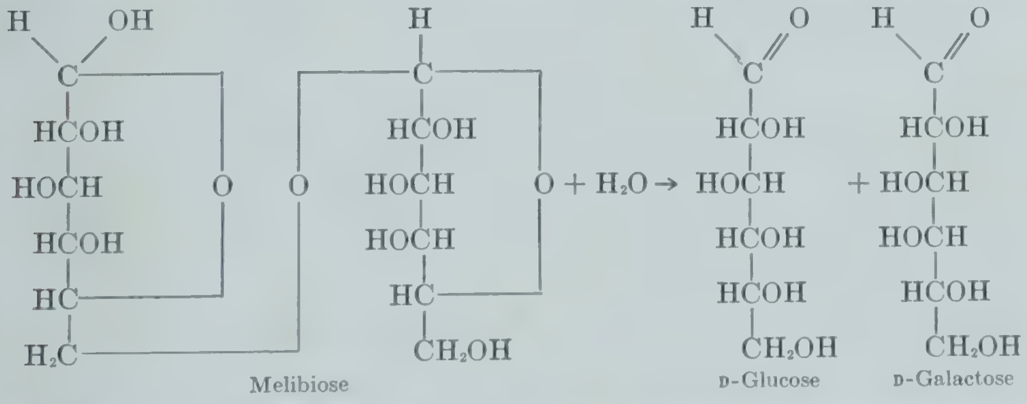
The ascosporeforming (sporogenous) yeasts have been classified b Stelling-Dekker (1931); and the nonascosporeforming (asporogenous, o anascosporogenous) yeasts, by Lodder (1932, 1934) and by Diddens an Lodder (1942). Individual genera have been considered by Custer (1940), who studied the genus *Brettanomyces;* by Bedford (1942), wh made a taxonomic study of the genus *Hansenula;* by Nickerson (1944) who studied the genus *Zygosaccharomyces;* by Wickerham (1951), wh studied the genus *Hansenula;* by Phaff (1956), who made a proposal fo the amendment of the diagnosis of the genus *Pichia* Hansen; and b others.

Information concerning classification may be found in a number o sources other than those mentioned above. The comprehensive review of Henrici (1941) and of Skinner (1947), who considered the yeast-lik fungi (*Candida* and *Brettanomyces*), are particularly noteworthy. Valuable data are to be found in "Zymologia Medica," by Mackinnon (1946); and in "Henrici's Molds, Yeasts and Actinomycetes," by Skinner, Emmons, and Tsuchiya (1947).

According to Schultz and Atkin (1947), the bios requirements of different strains of yeast may be used as a basis for classifying them. In order to determine the bios requirements, the yeast is grown in a special synthetic medium which contains casein hydrolyzate and growth factors. The latter are numbered as follows: (1) inositol, (2) calcium pantothenate, (3) biotin, (4) thiamin, (5) pyridoxine, (6) thiamin and pyridoxine, and (7) nicotinic acid. The bios requirement is placed as a number after the species name of the yeast. For example, *S. cerevisiae* 23 indicates that this particular strain requires calcium pantothenate and biotin for full growth.

**The Fermentation of Raffinose and Melibiose.** The ability of certain yeasts to attack raffinose and melibiose, or their inability to attack them, has been used as a differential test and as an aid in classification; for example, Stelling-Dekker used this test in classifying the genus *Saccharomyces.*

Raffinose is a trisaccharide that structurally is a combination of melibiose and sucrose with the glucose radical common to the two. Upon partial hydrolysis by sucrase it is converted to melibiose and fructose, the enzyme sucrase acting only on the sucrose portion of the trisaccharide. Melibiose (glucopyranose-6-alpha-galactopyranoside) is a disaccharide, which may be converted by the enzyme melibiase to glucose and galactose.



Yeasts, which secrete both sucrase and melibiase, hydrolyze raffinose to 1 molecule each of fructose, glucose, and galactose. Such yeasts completely ferment raffinose. Other yeasts, which possess sucrase but not melibiase, hydrolyze raffinose to melibiose and fructose. These yeasts ferment only fructose or one-third of the raffinose molecule.

A number of procedures have been devised to determine the extent to which raffinose is fermented. These involve the use of a quantitative apparatus, such as the van Iterson–Kluyver apparatus (Henrici, 1941); the absorption of the carbon dioxide produced during fermentation by means of barium hydroxide and the titration of the excess alkali with a standard acid solution; the titration of the unfermented sugar after 14 days; and a method devised by Wickerham (1943), which is described below.

In Wickerham's procedure, a 4 per cent nutrient solution of raffinose is dispensed in special tubes and inoculated with the yeast being tested. After the fermentation has subsided and the volume of gas has commenced to decrease, each tube is inoculated with a melibiose-fermenting yeast. A record is made of the amount of gas produced by each yeast.

The basal medium contained 0.45 per cent Difco yeast extract, 0.75 per cent peptone, and sufficient bromothymol blue to produce a green color at a pH of 7. Two-ml. portions of this medium were added to each of a series of culture tubes measuring 12 by 150 mm. and containing inverted vials measuring 6 by 50 mm. The tubes and their contents were sterilized, and to each tube was added aseptically 1 ml. of a 12 per cent solution of raffinose, sterilized for 20 min. at 12 lb. steam pressure. This medium was then inoculated with a young culture of the yeast being tested, which had been grown on a yeast extract agar slant. The tubes were incubated at 30°C. Gas production was recorded from time to time, and when the amount of gas in the inverted vial commenced to decrease, the tube was inoculated with a young culture of *S. carlsbergensis* NRRL 379, an active fermentor of melibiose. The production of additional gas indicated that the test yeast had not fermented melibiose. However, failure of *S. carlsbergensis* to produce additional gas was taken as evidence that the test yeast had already fermented melibiose.

Wickerham, in surveying 200 strains of industrial yeasts, found that the average length of time required for a yeast to complete the fermentation and for the volume of the gas entrapped in the inverted vial to commence to decrease was 7 days and that the longest time required was 16 days.

Henrici used culture tubes of 25- by 100-mm. size containing 20 ml. of medium and inverted Wassermann tubes (10 by 75 mm.) as gas traps in carrying out the melibiose fermentation test. He suggested that tubes inoculated with pellicle-forming yeasts be shaken a day or two after inoculation in order that some of the cells might sink to the bottom, and also that all tubes be shaken before reading the final results.

**Auxanographic Technique.** In order to determine whether or not a yeast is able to utilize a given sugar, Beijerinck (1889) devised an auxanographic procedure. The basal medium, which contained 0.1 per cent potassium dihydrogen phosphate, 0.05 per cent magnesium sulfate, 0.05 per cent ammonium sulfate, and 2 per cent washed agar, was prepared, sterilized, cooled, seeded heavily with the yeast being tested while still in the melted condition, and poured into petri dishes. The heavy suspension of yeast was used in order to provide the required growth-promoting substances. Small amounts of the sugars to be tested (in the dry form) were placed upon the dry surface of the solidified agar, sufficiently far apart to prevent mixing of the dissolved materials. Glucose was used as a control, since all yeasts are able to utilize it. The petri dishes were incubated at an optimum temperature for growth, usually 25 to 28°C. The sugars diffuse into the agar and, if they are assimilated, cause growth of the yeasts. No growth takes place when a sugar cannot be utilized. This procedure has been used by Lodder and others.

Lodder (1934) used a modification of Beijerinck's auxanographic technique in order to determine the extent to which certain nitrogen compounds were used by yeasts. The basal medium in this case contained 2 per cent glucose, 0.1 per cent potassium dihydrogen phosphate, 0.05 per cent magnesium sulfate, and 2 per cent washed agar. In carrying out the test, approximately 2 ml. of a heavy suspension of the yeast being examined was placed in each petri dish and the basal medium added. The plates were incubated at 30°C. for a number of hours in order to dry the surface of the agar. Small amounts of the nitrogen compounds (ammonium sulfate, asparagine, potassium nitrate, peptone, and urea) were then placed on the surface of the agar. The petri dishes were incubated at 25°C. and later observed to determine which sources of nitrogen supported growth.

Since the auxanographic method may produce erroneous results if a sugar or nitrogen compound diffuses over too wide an area in the petri dish, some workers have preferred to use liquid media and to test for the utilization of each given substance in separate culture tubes. Likewise, since a number of yeasts are studied at one time in modern investigations, one or more plates are prepared to contain one sugar or one nitrogen source only. The replica-plating technique may then be used.

**Replica-plating Technique.** A method of replica plating was developed by Lederberg and Lederberg (1952). The application of this method to the study of carbon assimilation by yeasts was described by Shifrine, Phaff, and Demain (1954). An outline of their procedure follows.

The yeast cultures are grown for 18 hr. on wort agar slants before being transferred to an initial plate, which may contain (1) wort agar or (2) yeast nitrogen base (Difco) with 0.5 per cent glucose and 2 per cent agar added. Enough yeast inoculum is used to produce pinpoint colonies (before incubation) on the plates. The inoculated plates, each containing 25 pinpoint colonies, are incubated at 22 to 25°C. for 12 to 16 hr. After incubation, each plate is inverted and pressed against a sterile velveteen fabric, previously mounted on a wooden block. The test plates (which contain a 0.5 per cent concentration of the carbon source, yeast nitrogen base, and purified agar) are then inverted successively on the block, pressed gently against the fabric, and lifted straight upward (to avoid smearing of the colonies). The order of replication is (1) control plate containing no carbon source, (2) sucrose, (3) maltose, (4) lactose, (5) galactose, and (6) glucose. The plates are examined after incubation at 22 to 25°C. for 2 to 6 days.

According to the authors, 6 to 10 successive replications may be made by the velveteen-block method from a plate containing 25 different colonies of yeasts.

The technique saves considerable time when a large number of yeasts must be identified.

A diagrammatic illustration of the replica-plating technique is shown in Fig. 8.

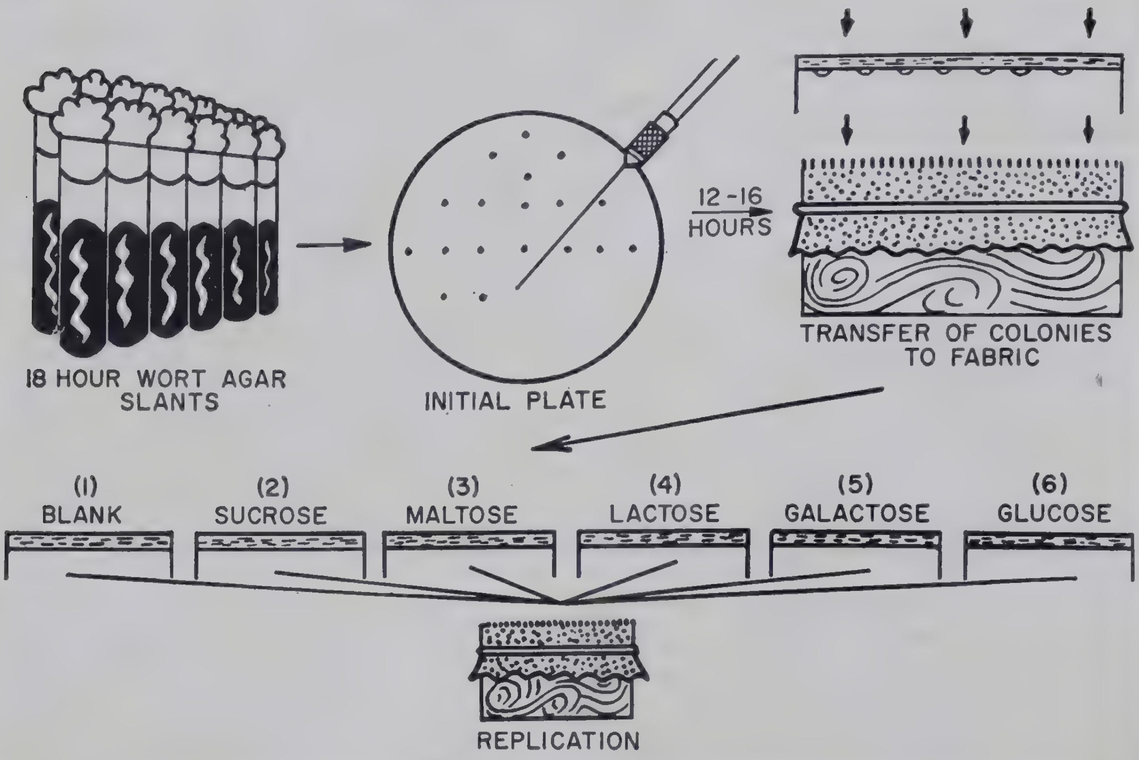


Fig. 8. Illustration of replica-plating technique for determination of sugar assimilation by yeasts. [*Courtesy of M. Shifrine, H. J. Phaff, and A. L. Demain, Jour. Bact.*, **68**: 28 (1954)].

**The Multipoint Inoculator.** This apparatus, described by Beech, Carr, and Codner (1955), is comprised of an inoculum tube holder, a multipoint inoculator, and a holder for a petri dish, as shown in Fig. 9. It is particularly useful for inoculating replicate agar plates with a maximum of 24 strains of yeasts or bacteria. Phaff (1956) reported that a slightly modified model of this inoculator was "vastly superior" to the velveteen-cloth method developed by his laboratory for studying the carbon-assimilation reactions of yeasts, that identical inocula were secured each time, and that the "smearing" problem was avoided.

**The Nutrition of Yeasts.** Yeasts, like bacteria and other forms of life, require certain food materials and environmental conditions for proper growth and reproduction. Some elements are basically necessary, as, for example, carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium, sulfur, calcium, iron, and magnesium. There is accumulating evidence which indicates that trace elements also play an important role in nutrition. Vitamins and/or other organic compounds are required for the satisfactory development or functioning of most types of yeasts. An adequate supply of water is essential for carrying out their life activities.

*Carbon.* In considering sugars as carbon sources, one must be reminded that the ability of a yeast to assimilate a sugar may be quite different from its ability to ferment the same sugar. Likewise the ability to assimilate a given compound varies with different varieties of yeasts.

Carbon may be supplied in the form of sugars, salts of some organic acids, glycerol, ethanol, and, occasionally, in other form, depending upon

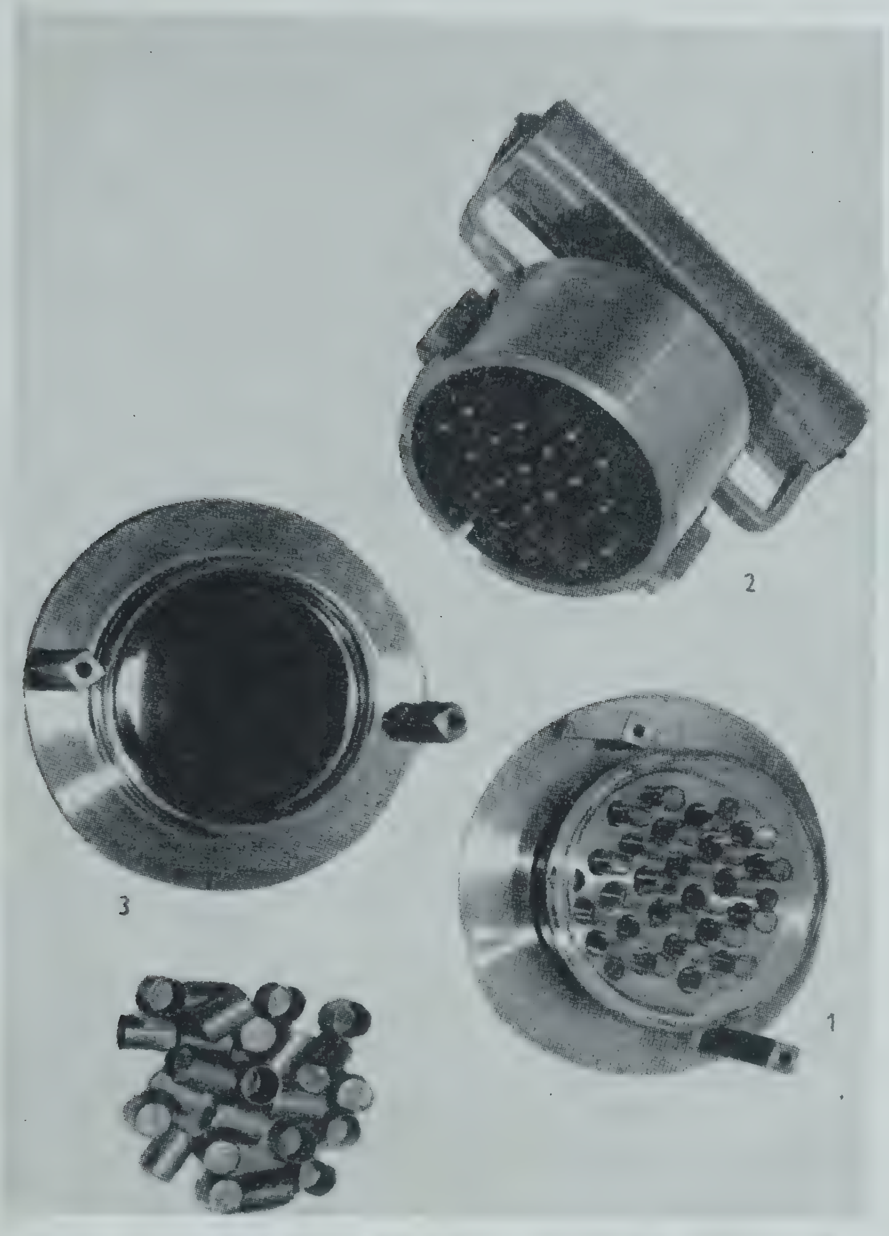


FIG. 9. A multipoint inoculator. [*Courtesy of F. W. Beech, J. G. Carr, and R. C. Codner, Jour. Gen. Microbiol.,* **13**(3): 408 (1955).]

the yeast. No species of yeasts are known which are able to assimilate cellulose or hemicellulose, and only a few industrially unimportant species secrete the amylases required to utilize starches.

Glucose, fructose, and mannose are assimilated well by most yeasts; sucrose and maltose are assimilated by many fewer species; and lactose is assimilated only in a few cases. The abilities of certain species of yeasts to assimilate some of the better-known sugars are indicated in Tables 4 and 5.

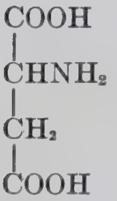
Acetates, citrates, lactates, malates, succinates, and tartrates, as well as lactic, malic, succinic, and tartaric acids; ethyl alcohol in low concentration; and certain other substances may serve as carbon sources.

*Nitrogen.* This element may be supplied to yeasts, depending on the species or strain, in the form of ammonia, ammonium salts (such as ammonium sulfate and diammonium hydrogen phosphate), amino acids, peptides, peptones (or other soluble protein derivative), urea, or nitrate. Only a relatively few species of yeasts are able to assimilate nitrogen from nitrates. Species of the genus *Hansenula* are able to utilize nitrates.

Ammonia and ammonium salts, particularly ammonium sulfate and diammonium hydrogen phosphate, appear to be most suitable sources of nitrogen on account of their availability, low cost, and ready assimilation. Ammonia is apparently preferred by yeasts to other sources of nitrogen when these are present in a medium. Amino acids are deaminized, and the ammonia is used by the yeasts.

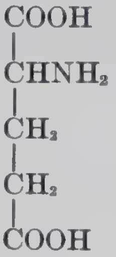
Wort, which contains amino acids and other soluble nitrogen compounds (produced by the action of the proteolytic enzymes of malt on the proteins of barley), is an adequate and good source of nitrogen (refer to the chapter on Brewing).

UTILIZATION OF AMINO ACIDS. There is considerable difference among the amino acids in respect to their availability and value as nitrogen sources for yeasts. The difference depends in part upon the position of the amino group present and the isomeric form available. In most cases where the amino group is attached to the carbon atom adjacent to the carboxyl group (alpha position), the nitrogen from the amino group is utilized. For example, aspartic acid, glutamic acid, leucine, asparagine, glycine, and tyrosine are very good sources of nitrogen for yeasts. According to Nielsen (1937), 99 per cent of the nitrogen from aspartic acid, asparagine, and glycine is assimilated by yeasts. The structural formulas of these compounds follow:



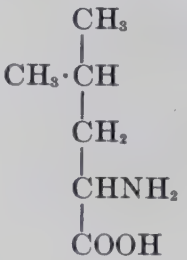
$$\begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{CHNH_2}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{COOH}\end{array}$$

Aspartic acid



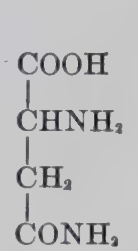
$$\begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{CHNH_2}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{COOH}\end{array}$$

Glutamic acid



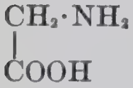
$$\begin{array}{rl} & \mathrm{CH_3}\\ & |\\ \mathrm{CH_3}\cdot & \mathrm{CH}\\ & |\\ & \mathrm{CH_2}\\ & |\\ & \mathrm{CHNH_2}\\ & |\\ & \mathrm{COOH}\end{array}$$

Leucine



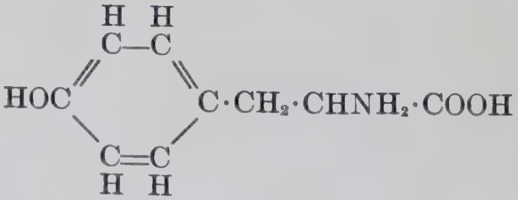
$$\begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{CHNH_2}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{CONH_2}\end{array}$$

Asparagine



$$\begin{array}{l}\mathrm{CH_2\cdot NH_2}\\ |\\ \mathrm{COOH}\end{array}$$

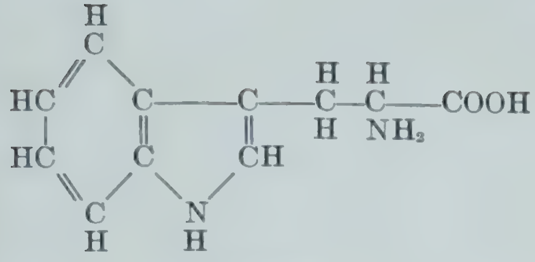
Glycine



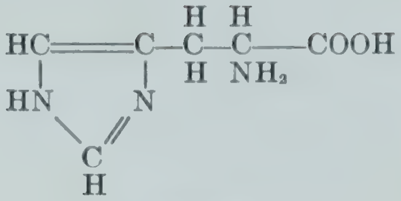
Tyrosine

In some instances only a fraction of the total nitrogen is utilized. Only about one-half (56 per cent) of the nitrogen of tryptophan and one-third (34 per cent) of the nitrogen of histidine are used, indicating that the alpha-amino group is attacked (Burk and Horner, 1939).

All amino acids except glycine exist in two isomeric forms (the dextro and levo forms). Yeasts appear to be able to utilize the form of amino acid that occurs naturally in preference to the synthetic isomer but will utilize both isomers of some amino acids, for example, the dextro and levo forms of aspartic and glutamic acids and asparagine.



Tryptophan

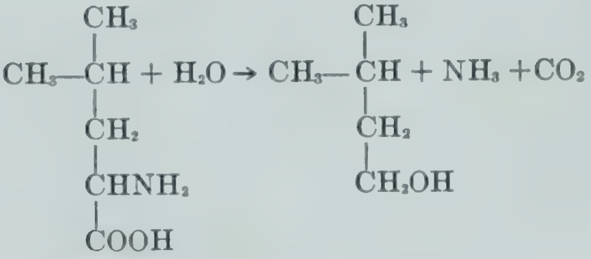


Histidine

CONVERSION OF AMINO ACIDS TO ALCOHOLS. Ehrlich has shown that certain amino acids, namely, leucine, isoleucine, phenylalanine, tryptophan, and tyrosine, are converted to alcohols by yeasts. His work has been confirmed by Thorne. The reaction is a hydrolytic deamination and decarboxylation. The amino group is hydrolyzed and the carboxyl group is decarboxylated in accordance with the following general reaction:

$$\underset{\text{Amino acid}}{R\cdot CHNH_2\cdot COOH} + H_2O \rightarrow \underset{\text{Alcohol}}{R\cdot CH_2OH} + NH_3 + CO_2$$

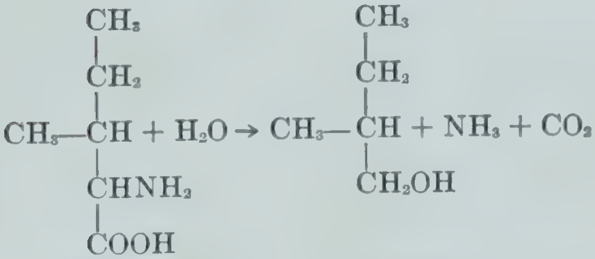
The reactions for the conversion of certain amino acids to alcohols follow:



$$\begin{array}{r@{}l} & CH_3 \\ & | \\ CH_3- & CH \\ & | \\ & CH_2 \\ & | \\ & CHNH_2 \\ & | \\ & COOH \end{array} + H_2O \rightarrow \begin{array}{r@{}l} & CH_3 \\ & | \\ CH_3- & CH \\ & | \\ & CH_2 \\ & | \\ & CH_2OH \end{array} + NH_3 + CO_2$$

Leucine

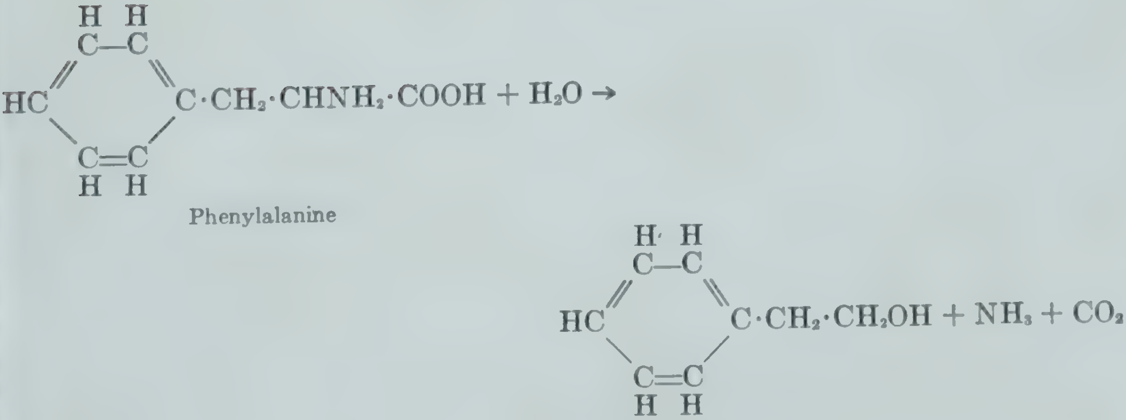
Isoamyl alcohol



$$\begin{array}{r@{}l} & CH_3 \\ & | \\ & CH_2 \\ & | \\ CH_3- & CH \\ & | \\ & CHNH_2 \\ & | \\ & COOH \end{array} + H_2O \rightarrow \begin{array}{r@{}l} & CH_3 \\ & | \\ & CH_2 \\ & | \\ CH_3- & CH \\ & | \\ & CH_2OH \end{array} + NH_3 + CO_2$$

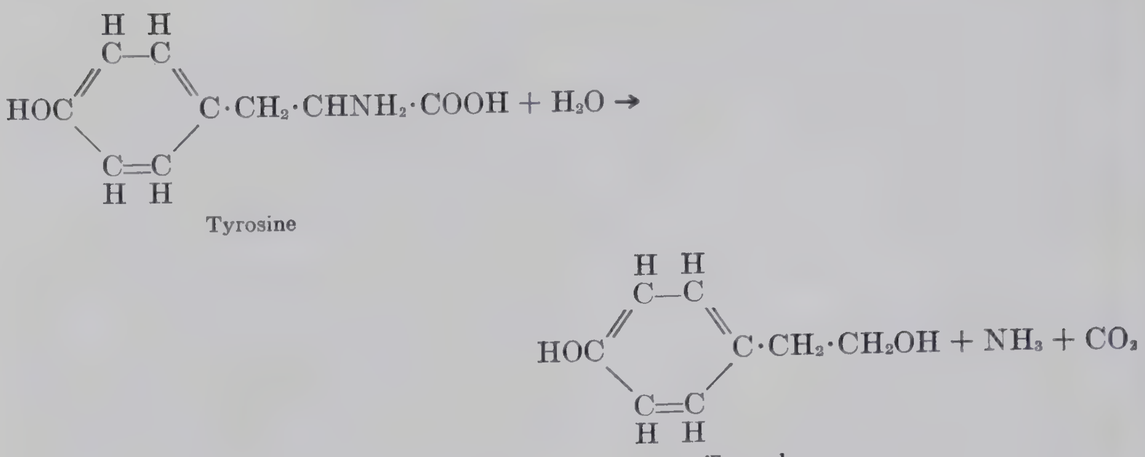
Isoleucine

Amyl alcohol



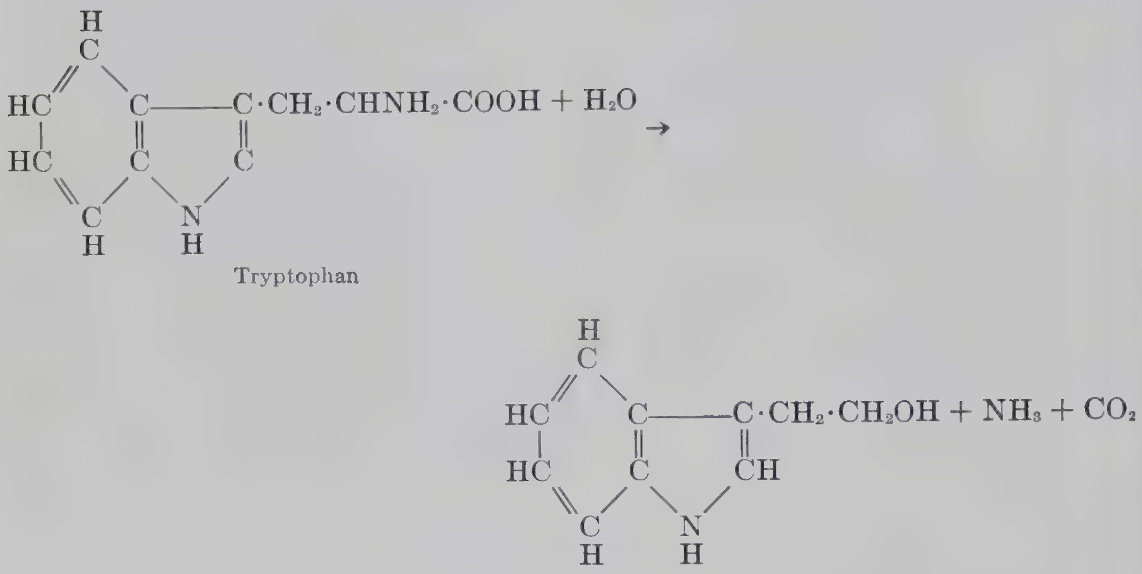
Phenylalanine

Phenylethyl alcohol



Tyrosine

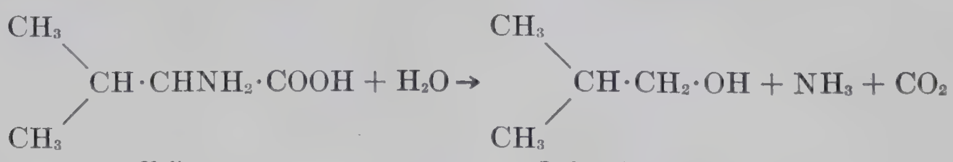
Tyrosol



Tryptophan

Tryptophol

Thorne (1937) has shown that isobutyl alcohol may be formed from valine by yeast in accordance with the following reaction:



$$\begin{matrix} CH_3 & & & CH_3 & \\ & \diagdown & & & \diagdown \\ & & CH\cdot CHNH_2\cdot COOH + H_2O \rightarrow & & & CH\cdot CH_2\cdot OH + NH_3 + CO_2 \\ & \diagup & & & \diagup \\ CH_3 & & & CH_3 & \end{matrix}$$

Valine

Isobutyl alcohol

The ammonia liberated by any of the foregoing reactions is available for utilization by yeasts. The reactions explain the origin of some of the constituents of fusel oil, such as amyl alcohol, isoamyl alcohol, and isobutyl alcohol.

EFFECT OF VITAMINS ON NITROGEN ASSIMILATION. The ability or the inability of a given species of yeast to utilize a particular nitrogen source has been used as one means of classifying yeasts. Stelling-Dekker (1931) employed potassium nitrate in classifying the sporogenous yeasts. Later, Lodder (1934) used additionally ammonium sulfate, asparagine, peptone, and urea as aids in the classification of asporogenous yeasts. In order to determine the ability of the yeasts to utilize a particular nitrogen compound, she used a modification of the auxanographic method. A large inoculum of yeast was employed to supply the growth-promoting substances required.

Wickerham (1946) carried out tests with various species of yeasts reputedly unable to utilize ammonium sulfate, urea, and asparagine. He demonstrated that the strains tested (obtained from the Northern Utilization Research Branch stock collection) were able to utilize all three of these nitrogen compounds when his special medium containing vitamins and certain trace elements was used. He also showed that urea in 0.092 per cent concentration inhibited the growth of all but 1 of the 10 species of *Candida* tested. However, urea in a concentration of 0.046 per cent was assimilated by all 10 species.

The results obtained by Wickerham are of great significance, particularly in respect to the classification of yeasts, for they show that, in the presence of vitamins, yeasts may assimilate nitrogen sources which they otherwise might not.

Wickerham devised and used the following medium (shown in Table 13), which includes trace elements and eight pure vitamins. This medium was used in both solid and liquid forms.

TABLE 13. COMPOSITION OF WICKERHAM'S SPECIAL MEDIUM *

| Ingredients | Concentration | Ingredients | Concentration |
|---|---|---|---|
| Trace elements: | | Vitamins: | |
| Boron, as $H_3BO_3$ | 0.01 p.p.m. | Biotin | 2 $\mu$g.† |
| Copper, as $CuSO_4 \cdot 5H_2O$ | 0.01 p.p.m. | Calcium pantothenate | 400 $\mu$g. |
| Iodine, as KI | 0.10 p.p.m. | Inositol | 2,000 $\mu$g. |
| Iron, as $FeCl_3 \cdot 6H_2O$ | 0.05 p.p.m. | Niacin | 400 $\mu$g. |
| Zinc, as $ZnSO_4 \cdot 7H_2O$ | 0.07 p.p.m. | *p*-Aminobenzoic acid | 200 $\mu$g. |
| | | Pyridoxine hydrochloride | 400 $\mu$g. |
| | | Thiamin hydrochloride | 400 $\mu$g. |
| | | Riboflavin | 200 $\mu$g. |
| Salts: | | Carbon source: | |
| $KH_2PO_4$ | 0.875 g.† | Glucose | 10.0 g.† |
| $K_2HPO_4$ | 0.125 g. | | |
| $MgSO_4 \cdot 7H_2O$ | 0.50 g. | Nitrogen sources: ‡ | |
| NaCl | 0.10 g. | $(NH_4)_2SO_4$ | 1.00 g.† |
| $CaCl_2 \cdot 2H_2O$ | 0.10 g. | $KNO_3$ | 0.78 g. |
| | | Urea | 0.46 g. |
| | | Asparagine | 1.00 g. |
| | | Peptone | 1.32 g. |

* L. J. Wickerham, *Jour. Bact.*, **52**(3): 293 (1946).

† Units given in terms of a liter of solution.

‡ Used separately.

*Mineral Requirements.* The exact mineral requirements of yeasts in many cases are not fully known. The literature contains some conflicting statements on the subject, as has been shown by Joslyn (1941) in his extensive review of the mineral metabolism of yeasts. Further careful research in this field should lead to a better understanding of the subject and practical improvements in the selection of media for growth, sporulation, and fermentation.

Chemical and spectroscopic analyses of yeasts and the media in which they are grown furnish considerable valuable information regarding the indispensability of the elements. However, the mere fact that an element is found in the ash of yeast in small amounts does not establish its essentiality.

Table 14 presents data concerning the composition of yeast ashes as determined by three different laboratories.

TABLE 14. COMPOSITION OF YEAST ASHES *

| Ash constituent | Top yeast, % † | Bakers' yeast, % ‡ | Bakers' yeast, % § |
|---|---|---|---|
| $P_2O_5$ | 52.3 | 52.3 | 54.5 |
| $K_2O$ | 35.4 | 35.4 | 36.5 |
| $Na_2O$ | 0.06 | 0.60 | 0.7 |
| MgO | 4.8 | 4.8 | 5.2 |
| CaO | 1.56 | 1.56 | 1.4 |
| $SiO_2$ | 1.1 | 1.1 | 1.2 |
| $SO_3$ | 0.41 | .... | 0.5 |
| Cl | .... | .... | Trace |
| FeO | 0.43 | 0.34 | Trace |

* M. A. Joslyn, *Wallerstein Labs. Communs.,* **4**(11): 49 (1941).
† Fulmer et al. (1928).
‡ Heller (1925).
§ Frey (1930).

A study of this table indicates that phosphorus and potassium are present in large amounts in yeast ash, comprising about 90 per cent of the total elements. The elements magnesium, calcium, silicon, sodium, iron, and sulfur are present in considerably smaller quantities.

Phosphorus is a particularly important element in the life processes of yeasts. It plays a very significant role in the production of ethyl alcohol from sugars, as, for example, in the formation of hexose and triose phosphates. It is a component of cozymase and of cocarboxylase (an activator of the enzyme carboxylase). It is found in nucleic acid, in lecithin, and in other components of the yeast cell.

Natural media and worts normally contain sufficient of the inorganic elements for good growth. However, in studying the effect of a given element or growth-promoting substance on growth or fermentation, it is in most cases essential to know the exact composition of the medium employed. Ordinary chemical analysis often fails to reveal sufficient information concerning the trace elements present. Such information may be supplied by the use of a spectroscope. Richards and Troutman (1940) have analyzed spectroscopically the mineral content of the ashes from yeasts grown in certain culture media, and of the ashes from the components of the media. Table 15 presents data obtained by them.

TABLE 15. ELEMENTS PRESENT IN YEAST ASHES AND IN THE COMPONENTS OF THE CULTURE MEDIA *, †

| Element | Ashes from yeast grown in | | | | | Ashes from components of media | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Williams' medium | | Malt extract | Yeast extract | Autolyzed yeast | Asparagine | Asparagine mother liquor | $(NH_4)_2SO_4$ | $KH_2PO_4$ | $CaCl_2$ | $MgSO_4$ | Malt extract |
| | Standard | Enriched | | | | | | | | | | |
| Al | − | − | − | − | ++ | + | + | − | − | − | − | − |
| Ba | + | + | + | + | ? | + | + | + | − | + | − | + |
| Be | − | − | − | − | ? | − | − | − | − | − | − | − |
| Bi | − | + | ? | + | + | − | − | − | ? | − | − | − |
| B | − | − | ++ | ++ | ++ | ++ | ++ | − | ? | − | − | ++ |
| Ca | + | + | + | + | + | + | + | ? | ? | ‡ | − | + |
| Cr | + | + | + | + | + | + | + | + | + | − | − | + |
| Cu | ++ | ++ | ++ | ++ | ++ | ++ | ++ | − | − | − | − | ++ |
| Au | + | + | + | + | ? | + | + | − | + | − | − | + |
| Fe | + | + | + | + | + | + | + | + | + | + | − | + |
| La | + | + | + | − | ? | − | + | − | − | − | − | + |
| Pb | + | + | ++ | − | + | + | + | ? | − | − | − | ? |
| Li | − | − | − | − | − | + | ? | − | − | + | − | + |
| Mg | + | + | + | + | + | + | + | − | − | − | ‡ | + |
| Mn | ++ | + | ++ | ++ | ++ | ++ | ++ | − | − | − | ? | ++ |
| P | + | + | + | + | + | + | + | − | ‡ | − | − | + |
| K | + | + | + | + | + | + | + | − | ‡ | − | − | + |
| Rb | ? | ? | ? | − | − | ? | ? | − | − | − | − | ? |
| Pt | + | + | + | − | + | + | + | − | − | − | − | − |
| Si | + | − | ++ | ++ | ++ | ++ | ++ | − | − | − | − | + |
| Ag | + | + | + | + | + | + | + | − | ? | ? | − | − |
| Sr | − | − | + | ++ | + | + | ++ | − | − | + | − | + |
| Tl | + | ++ | + | ? | + | + | + | − | − | − | − | − |
| Sn | + | ++ | ++ | ++ | ++ | ++ | ++ | ? | + | − | − | + |
| W | − | − | − | − | − | ? | ? | − | − | ? | − | ? |
| V | − | ? | − | ? | ? | − | − | − | − | − | − | − |
| Zn | ? | − | + | − | − | + | ++ | − | − | − | − | ? |

* O. W. Richards and M. C. Troutman, *Jour. Bact.*, 39(6): 739 (1940).

† + = present, ++ = strongly present, − = absent, ? = possible trace.

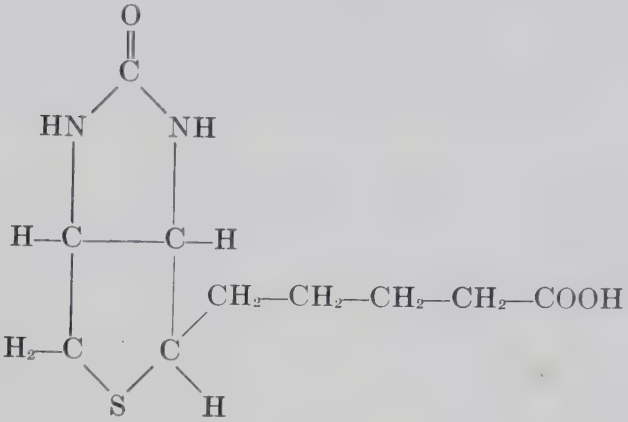
‡ Known part of medium.

Certain trace elements, for example, copper, iron, thailium, and manganese, are of considerable importance. Elvehjem (1931) demonstrated hat bakers' yeast will grow poorly in a medium low in iron and copper. hese elements appear to be essential for the elaboration of cytochrome. ron catalyzes respiration through the action of catalase, peroxidase, and ytochrome oxidases. According to Richards (1925), growth of yeast is timulated when thallium is added to Williams' medium (20 g. of sucrose, g. of $(NH_4)_2SO_4$, 2 g. of $KH_2PO_4$, 1.5 g. of asparagine, 0.25 g. of $CaCl_2$, nd 0.25 g. of $MgSO_4 \cdot 7H_2O$ in 1 liter of distilled water). Edbacher is of e opinion that manganese activates yeast arginase.

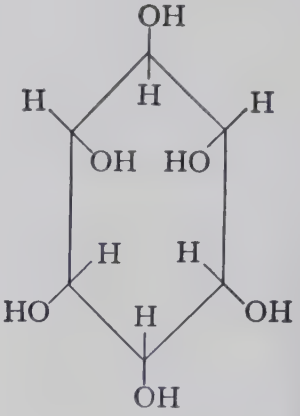
*Vitamins.* Various vitamins of the B group are necessary for th growth of yeasts. Individual requirements differ, however.

Table 16 shows the structural formulas of vitamins which, for th most part, are important in the nutrition of yeasts.

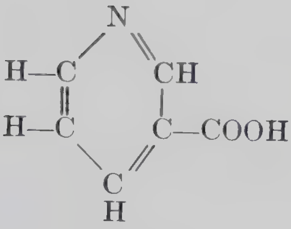
Table 16. Structural Formulas of Vitamins Important in the Nutrition of Yeasts



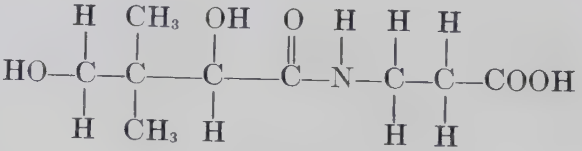
Biotin



Myo-inositol

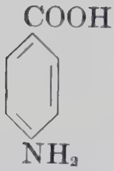


Nicotinic acid

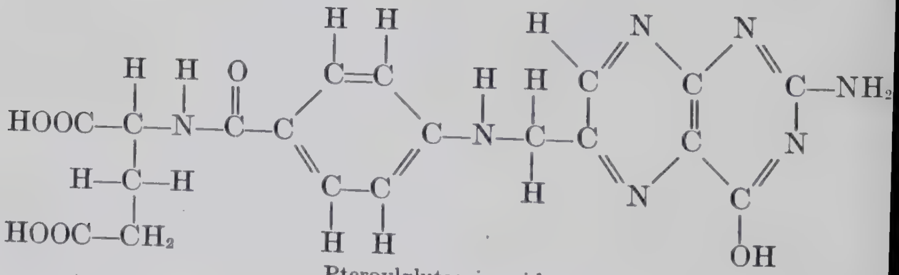


$$\mathrm{HO{-}\overset{H}{\underset{H}{C}}{-}\overset{CH_3}{\underset{CH_3}{C}}{-}\overset{OH}{\underset{H}{C}}{-}\overset{O}{\overset{\|}{C}}{-}\overset{H}{N}{-}\overset{H}{\underset{H}{C}}{-}\overset{H}{\underset{H}{C}}{-}COOH}$$

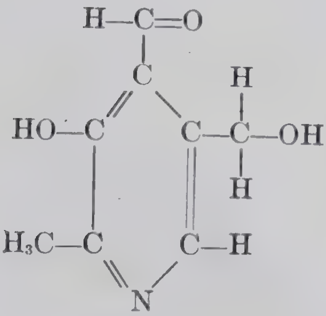
Pantothenic acid



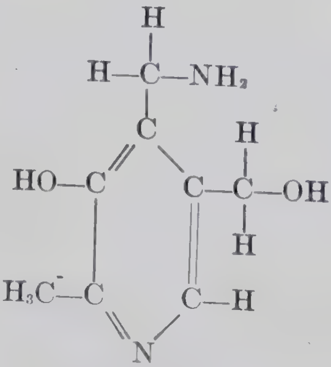
*p*-Aminobenzoic acid



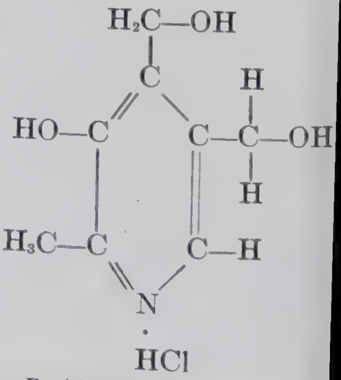
Pteroylglutamic acid



Pyridoxal

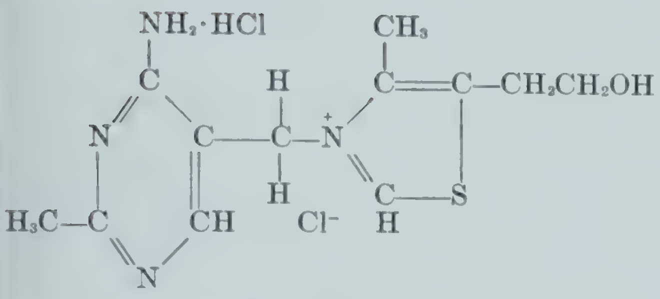


Pyridoxamine

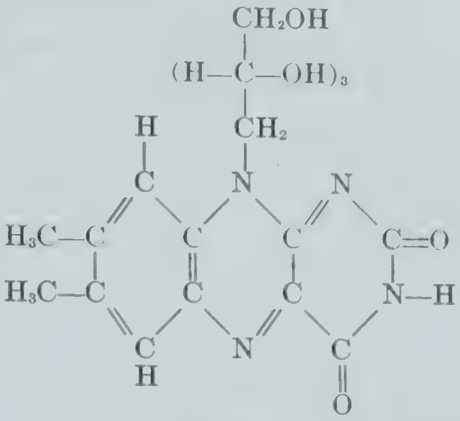


Pyridoxine hydrochloride

TABLE 16. STRUCTURAL FORMULAS OF VITAMINS IMPORTANT IN THE NUTRITION OF YEASTS (*Continued*)



Thiamin hydrochloride



Riboflavin

---

**Media.** Food materials are supplied to yeasts in the form of media (singular, medium) which contain all the essential materials necessary for growth, reproduction, or fermentation. Media may be synthetic, semisynthetic, or nonsynthetic in nature. A synthetic medium is one in which the composition of all the ingredients is known. A semisynthetic medium is one containing some constituents of known composition and some whose analysis is only approximately known. A nonsynthetic medium is one whose exact composition is not known. The composition of chemically pure salts, sugars, acids, and certain other chemical compounds is definitely established, whereas that of such items as beef extract and peptone is more qualitative than quantitative. Media such as apple juice and grape juice are natural sources of the required food materials for the yeasts, as is evident by the fact that the yeasts found on the surfaces of apples and grapes rapidly ferment at optimum temperatures the juices expressed from them.

Wickerham's yeast morphology agar (and his carbon and nitrogen bases), potato dextrose agar, wort agar, and malt extract broth are a few of the media which are available commercially in dehydrated form.

**Flocculation of Yeasts.** Flocculation is a phenomenon concerned with the separation of yeast cells from the suspending substrate. The cells accumulate at the top of the mash in the case of top-fermentation yeasts and settle as a sediment in the case of bottom-fermentation yeasts.

Depending on the time of occurrence, flocculation may be an asset or a cause of concern. The flocculation which occurs near the end of fermentation in the brewery is valuable; however, the flocculation which takes place too early or too readily may result in incomplete attenuation of the wort, unless rousing is accomplished to remedy the situation. In the bakers' yeast industry, the too rapid settling of yeast cells from the aqueous suspension being prepared for incorporation into the dough is highly undesirable.

Yeasts may be classified on the basis of their relative flocculating bilities. Powdery, gritty, or nonflocculating strains are those which remain fairly uniformly dispersed in the wort during all the stages of the primary fermentation. Opposed to these strains are those which are strongly flocculating and which tend to flocculate early during the fermentation. Other strains of yeasts possess characteristics intermediate between these two extremes.

A classification of yeasts into four classes on the basis of flocculation characteristics was proposed by Gilliland (1951). Class I yeasts included those which were completely dispersed during all stages of fermentation Classes II, III, and IV included flocculating strains. Class IV yeasts flocculated very early during the fermentation, because the newly formed daughter cells did not separate. Classes II and III flocculated late during the fermentation; class II, into small, loose clumps; and class III, into dense masses. The cells of both of these classes were completely dispersed at the start of the fermentation.

A number of theories have been proposed to explain the cause or causes of flocculation. These have been reviewed by Dunn (1955). Some are listed in Table 17. Thorne (1951, 1952) and Gilliland (1951) showed that flocculence was a hereditary characteristic controlled by the genes (o gene). Both genetics and the electrical charge on the yeast cells seem t be significant factors in the control of flocculation. However, other factor exert some influence on this phenomenon.

TABLE 17. THEORIES CONCERNING CAUSES OF FLOCCULATION *

| Theory | Reference |
|---|---|
| Electrical charge | Geys, K.: *Jour. Inst. Brewing,* **28**: 47 (1922).<br>Stockhausen, F.: *Wochschr. Brau.,* **44**: 121 133 (1927). |
| pH | Schoenfeld, A., and H. Krumhaar: *Woch- schr. Brau.,* **35**: 342 (1918). |
| Sugar concentration | Burns, J. A.: *Jour. Inst. Brewing,* **43**: 3 (1937). |
| Injury to cells | Rohrer, E.: *Brewers Dig.,* **25**: 54 (1950). |
| Effects of other microorganisms | Barendrecht, H. P.: *Zentr. Bakt. Para sitenk.,* **7**: 623 (1901).<br>Beijerinck, M. W.: *Zentr. Bakt. Parasitenk* **20**: 641 (1908).<br>Malkow, A.: *Zentr. Bakt. Parasitenk.,* **90** 212 (1934). |
| Lime—phosphoric acid | Schoenfeld, A., and H. Krumhaar: *Woch schr. Brau.,* **35**: 302 (1908). |
| Protein precipitation | |
| Variation in cultural conditions | |

* G. G. Dunn, *Am. Brewer,* December, 1955.

Problems relating to flocculation are occasionally somewhat complex. Since flocculation characteristics are associated with yeast genetics, it is obvious that if one strain of yeast does not do what is wanted another strain possessing the desired characteristics should be selected. Likewise, it would be possible in many cases, when time and facilities permit, to produce hybrids which would function satisfactorily for a particular requirement. Once in a while, drastic changes in manufacturing conditions, such as raw materials, pH, and microbial contamination, may adversely affect the flocculation properties of a strain of yeast. Careful study and control of manufacturing procedures are usually most helpful in preventing and correcting such unfavorable situations.

Methods for measuring yeast flocculence have been devised by Burns (1937); Nielsen (1937); Jansen and Mendlik (1951); Helm, Nøhr, and Thorne (1953); and others.

The interested reader is urged to consult some of the references listed at the end of this chapter for more details on this subject.

**Phytochemical Reduction.** Yeast possesses strong reducing properties. A relatively large number of substances may be added to an actively fermenting medium, containing yeast and sugar, and become reduced. Aldehydes are reduced to their corresponding alcohols; for example, acetaldehyde, glyceraldehyde, furfuraldehyde (furfural), and benzaldehyde are reduced to ethyl alcohol, glycerol, furfuralcohol, and benzyl alcohol, respectively. Unsaturated aldehydes, such as citral and cinnamaldehyde, are reduced to their corresponding olefinic alcohols. Ketones, such as methylhexyl, methylnonyl, methylpropyl ketones, and acetylmethylcarbinol, and hydroxy- and diketones are reduced to their corresponding alcohols. Unsaturated alcohols are reduced to their corresponding saturated alcohols; nitrobenzene, to aniline; sulfur, to hydrogen sulfide; and oxidized methylene blue, to the reduced form. Quinones are reduced to quinols; and malic and tartaric acids, to succinic acid.

TABLE 18. SOME PRODUCTS FORMED IN WHOLE OR IN PART THROUGH THE ACTION OF YEASTS OR YEAST-LIKE MICROORGANISMS

| Acids | Alcohols | Alcohol-containing beverages | Coenzymes | Enzymes |
|---|---|---|---|---|
| Acetic | Amyl alcohol | Ales | Coenzyme I | Invertase |
| Digalacturonic | D-Arabitol | Beers | Coenzyme II | Lactase |
| Galacturonic | Ergosterol | Brandies | Coenzyme A | Lipase |
| Lactic | Ethyl alcohol | Kefir | | Polygalacturonase |
| Succinic | Glycerol | Kumiss | | |
| Tetragalacturonic | Isoamyl alcohol | Mead | | |
| | Mannitol | Perry | | |
| | Zymosterol | Rums | | |
| | | Whiskeys | | |
| | | Wines | | |

FOOD TECHNOLOGICAL RESEAR
LIBRARY

TABLE 18. SOME PRODUCTS FORMED IN WHOLE OR IN PART THROUGH THE ACTION OF YEASTS OR YEAST-LIKE MICROORGANISMS (*Continued*)

| Esters | Miscellaneous products * | Vitamins | Yeast products |
|---|---|---|---|
| Ethyl acetate, etc. | Adenylic acid | Biotin | Bakers' yeast |
| | Carbon dioxide | Folic acid | Brewers' yeast |
| | Fusel oil | Niacin | Fodder yeast |
| | Glutathione | *p*-Aminobenzoic acid | Food yeast |
| | Nucleic acid | Pyridoxine | Yeast extract |
| | Nucleotides | Riboflavin | Yeast gum |
| | Soy sauce | Thiamin | Yeast lipids |
| | Vinegars | | |

* In addition such products as amines, diketones, glycols, ketones, olefinic alcohols, quinols, and saturated alcohols may be formed by yeasts as reduction products.

Reduction may be demonstrated readily by adding the substance to be reduced to a fermentation medium containing 10 per cent sucrose and 10 per cent living yeast cells.

**Yeast Products.** Table 18 indicates some of the products which are formed in whole or in part through the action of yeasts. This is not a complete list, by any means, but it does include items of special interest.

### Some General References concerning Yeasts

Adams, A. M.: A Convenient Method of Obtaining Ascospores from Bakers' Yeast, *Can. Jour. Research,* **C, 27**: 179–189 (1949).

Adams, A. M., and J. J. Miller: Effect of Gaseous Environment and Temperature on Ascospore Formation in *Saccharomyces cerevisiae* Hansen, *Can. Jour. Bot.,* **32**: 320–334 (1954).

Agar, H. D., and H. C. Douglas: Studies of Budding and Cell Wall Structure of Yeast, *Jour. Bact.,* **4**: 427–434 (1955).

Barnett, J. A., and M. Ingram: Technique in the Study of Yeast Assimilation Reaction, *Jour. Appl. Bact.,* **18**: 131–148 (1955).

Bartholomew, J. M., and T. Mittwer: Demonstration of yeast bud scars with the electron microscope, *Jour. Bact.,* **65**: 272–275 (1953).

Beech, F. W., J. G. Carr, and R. C. Codner: A Multipoint Inoculator, *Jour. Gen Microbiol.,* **13**: 408 (1955).

Beijerinck, M. W.: L'auxanographie, ou la méthode de l'hydro diffusion dans la gélatin appliquée aux recherches microbiologique, *Arch. néerl. sci.,* **23**: 367–37 (1889).

Benham, R. W.: The Genus *Cryptococcus, Bact. Revs.,* **20**: 189–198 (1956).

Brown ,B. M.: Brewery Yeasts, *Jour. Appl. Bact.,* **18**: 196–203 (1955).

Bunker, H. J.: Yeast Production: The Choice of Fat-producing and Food Yeasts *Jour. Appl. Bact.,* **18**: 180–186 (1955).

Burk, D., and C. K. Horner: Nitrogen Assimilation by Yeast, a Consideration o Yeast Nutrition and Metabolism, *Wallerstein Labs. Communs.,* **2**(6): 5–2 (1939).

Carson, H. L., E. P. Knapp, and H. J. Phaff: III. The yeast flora of the natura breeding sites of some 71 species of Drosophila, *Ecology,* **37**(3): 538–544 (1956

Challinor, S. W.: Cider Yeasts and the Fermentation of Apple Juice, *Jour. App Bact.,* **18**: 212–223 (1955).

Chiao, J. S., and W. H. Peterson: Yeasts' Methionine and Cystine Contents, *Jou Agr. Food Chem.,* **1**(16): 1005–1008 (1953).

Cook, A. H. (ed.): "The Chemistry and Biology of Yeasts," Academic Press, Inc., New York, 1958.

de Becze, G. I.: Yeasts: I. Morphology, *Appl. Microbiol.*, **4**(1): 1–12 (1956).

Dunn, C. G.: Food Yeast, *Wallerstein Labs. Communs.*, **15**(48): 61–79 (1952).

———: Recent Developments in the Field of Industrial Microbiology and Their Possible Impact on Brewing Technology, *Proc. Soc. Brewing Chemists*, 1955, pp 65–79.

Eddy, A. A.: A New Experimental Method for the Study of Yeast Flocculation, *Jour. Appl. Bact.*, **18**: 165–167 (1955).

Elvehjem, C. A.: The Role of Iron and Copper in the Growth and Metabolism of Yeast, *Jour. Biol. Chem.*, **90**: 111–132 (1931).

Etchells, J. L., T. A. Bell, and I. D. Jones: Morphology and Pigmentation of Certain Yeasts from Brines and the Cucumber Plant, *Farlowia*, **4**(3): 265–304 (1953).

———, R. N. Costilow, T. A. Bell, and A. L. Demain: Control of Molds during the Enumeration and Isolation of Yeasts from Soil and Plant Material, *Appl. Microbiol.*, **2**(5): 296–300 (1954).

Frey, Charles N.: History and Development of the Modern Yeast Industry, *Ind. Eng. Chem.* **22**: 1154 (1930).

———, G. W. Kirby, and A. Schultz: Yeast: Physiology, Manufacture and Uses, *Ind. Eng. Chem.*, **28**: 879–884 (1936).

Gilliland, R. B.: The Examination of Brewery Yeasts, *Jour. Appl. Bact.*, **18**: 161–164 (1955).

———: The Flocculation of Brewing Yeast, *Wallerstein Labs. Communs.*, **20**(68): 41–71 (1957).

Graham, V. R., and E. G. Hastings: Studies on Film-forming Yeasts, *Can. Jour. Research*, **19**: 251–256 (1941).

Guilliermond, A.: "The Yeasts," translated and revised by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

———: "Clef dichotomique pour la détermination des levures," Librairie de François, Paris, 1928.

———: "La sexualité, le cycle de développement, la phylogénie, et la classification des levures d'après les travaux récents," Masson et Cie, Paris, 1937.

Haddad, S. A., and C. C. Lindegren: A Method for Determining the Weight of an Individual Yeast Cell, *Appl. Microbiol.*, **1**(3): 153–156 (1953).

Hansen, A.: "Jorgensen's Micro-organisms and Fermentation," 7th ed., Charles Griffin & Co., Ltd., London, 1957.

Hedrick, L. R., and G. C. Burke: Two New Yeasts from Hawaiian Fruit Flies, *Mycopathol. et Mycol. Appl.*, **6** (fasc. 2): 92–95 (1951).

Hopkins, R. H.: Amylase Systems in Brewery Yeasts, "European Brewery Convention—Proceedings of the Congress Baden-Baden," Elsevier Publishing Company, Amsterdam, 1955, pp. 52–64.

ouwink, A. L., and D. R. Kreger: Observations on the Cell Wall of Yeasts: An Electron Microscope and X-ray Diffraction Study, *Antonie van Leeuwenhoek Jour. Microbiol. Serol.*, **19**: 1–24 (1953).

oslyn, M. A.: The Mineral Metabolism of Yeast, *Wallerstein Labs. Communs.*, **4**(11): 49–65 (1941).

luyver, A. J., and M. Th. J. Custers: The Suitability of Disaccharides as Respiration and Assimilation Substrates for Yeasts which Do Not Ferment These Sugars, *Antonie van Leeuwenhoek Jour. Microbiol. Serol.*, **6**: 121–162 (1939, 1940).

reipe, H.: "Laboratoriumsbuch für die Brennerei und Hefeindustrie," 2d ed., W. Knapp Verlag, Düsseldorf, Germany, 1952.

etzschmar, Hermann: "Hefe und Alkohol sowie andere Gärungs produkte," Springer-Verlag, Berlin, Vienna, 1955.

ufer, L., D. R. Schwarz, and E. D. Stewart: Yeast: A Source of Biochemical Intermediates, *Jour. Agr. Food Chem.*, **1**(1): 91–95 (1953).

ufer, S., and R. Schwarz: "Yeast Fermentation and Pure Culture Systems," Schwarz Laboratories, Inc., New York, 1936.

Lederberg, J., and E. M. Lederberg: Replica Plating and Indirect Selection of Bacterial Mutants, *Jour. Bact.*, **63**: 399–406 (1952).

Lindegren, C. C., and G. Lindegren: Sporulation in *Saccharomyces cerevisiae, Botan Gaz.*, **105**(3): 304–316 (1944).

——— and ———: Eight Genes Controlling the Presence or Absence of Carbohydrate Fermentation in *Saccharomyces, Jour. Gen. Microbiol.*, **15**: 19–28 (1956)

———, M. A. Williams, and D. O. McClary: The Distribution of Chromatin in Budding Yeast Cells, *Antonie van Leeuwenhoek Jour. Microbiol. Serol.*, **22**: 1–2 (1956).

Lodder, J., and N. J. W. Kreger-van Rij: Classification and Identification o Yeasts, *Lab. Prac.*, **3**: 483–490 (1954); **4**: 20–24, 53–57 (1955).

Lukes, T. M., and H. J. Phaff: Characteristics of Trehalase in *Candida tropicalis Antonie van Leeuwenhoek Jour. Microbiol. Serol.*, **18**: 323–335 (1952).

Lund, Aage: Yeasts in Nature, *Wallerstein Labs. Communs.*, **19**(66): 221–236 (1956)

McWilliams, C. S., and M. S. Peterson (eds.): "Yeast—Its Characteristics, Growth and Function in Baker Products," National Academy of Sciences—National Research Council, Advisory Board on Quartermaster Research and Developmen Committee on Foods, Subcommittee on Cereal and Baked Products, Washington January, 1957.

Menzinsky, G.: On Factors Influencing the Metabolism and Growth of *Saccharomyces cerevisiae* (Top Yeast) under Aerobic Conditions, *Arkiv Kemi,* **2**(1): 1–94 (1950).

Miller, J. J.: Metabolism of Yeast Sporulation. II. Stimulation and Inhibition by Monosaccharides, *Can. Jour. Microbiol.*, **3**: 81–90 (1957).

———, and C. Halpern: The Metabolism of Yeast Sporulation. I. Effect of Certain Metabolites and Inhibitors, *Can. Jour. Microbiol.*, **2**: 519–537 (1956).

———, and N. S. Webb: Isolation of Yeasts from Soil with the Aid of Acid, Rose Bengal and Oxgall, *Soil Sci.*, **77**(3): 197–204 (1954).

Morris, E. O.: Microfermentation Tests and Colony Characters as Aids to Yeast Differentiation, *Jour. Appl. Bact.*, **18**: 126–130 (1955).

Mrak, E. M.: The World I Live In, *Food Technol.*, **11**: 541–552 (1957).

——— and L. S. McClung: Yeasts Occurring on Grapes and in Grape Products in California, *Jour. Bact.*, **40**: 395–407 (1940).

——— and H. J. Phaff: "Taxonomy and Morphology of Yeasts," University of California, Food Technology Division, Davis, Calif.

———, ———, and H. C. Douglas: A Sporulation Stock Medium for Yeasts and Other Fungi, *Science*, **96**(2497): 432 (1942).

———, ———, and R. H. Vaughn: Yeasts Occurring on Dates, *Jour. Bact.*, **43**(6): 689–700 (1942).

———, ———, ———, and H. N. Hansen: Yeasts Occurring in Souring Figs, *Jour Bact.*, **44**(4): 441–450 (1942).

———, R. H. Vaughn, M. W. Miller, and H. J. Phaff: Yeasts Occurring during the Fermentation and Storage of Green Olives, *Food Technol.*, **10**(9): 416–419 (1956)

Nakayama, T., G. Mackinney, and H. J. Phaff: Carotenoids in Asporogenous Yeasts *Antonie van Leeuwenhoek Jour. Microbiol. Serol.*, **20**: 217–228 (1954).

Neuberg, C.: The Biochemistry of Yeasts, *Ann. Rev. Biochem.*, **15**: 435–474, 685–687 (1946).

Nickerson, W. J.: *Zygosaccharomyces Acidifaciens:* A New Acetifying Yeast, *Mycologia,* **35**(1): 66–78 (1943).

———: Some Trends in Research on Yeasts, *Chronica Botan.*, **7**(8): 409–412 (1943)

———: Studies in the Genus *Zygosaccharomyces*. I. Transfer of Pellicle-forming yeasts to *Zygopichia, Farlowia,* **1**(3): 469–481 (1944).

———: Studies on Film-forming Yeasts. Acid Production by *Zygopichia* and *Zygohansenula, Mycologia,* **36**(3): 224–233 (1944).

——— and W. R. Carroll: On the Metabolism of *Zygosaccharomyces, Arch. Biochem* **7**(1): 257–271 (1945).

Nielsen, N.: *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **21**: 395 (1936); *Sé chim.*, **22**: 384 (1937).

——. and V. Hartelius: The Separation of Growth Promoting Substances, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.,* **19**(8) (1932).

—— und ———: Über die Trennung der auf die Stoffproduktion der Hefe und Schimmelpilzen einwirkenden Wuchsstoffe, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.,* **22**(1) (1937).

orthcote, D. H., and R. W. Horne: *Biochem. Jour.,* **51**: 232 (1952).

terson, W. J., T. A. Bell, J. L. Etchells, and W. W. G. Smart, Jr.: A Procedure for Demonstrating the Presence of Carotenoid Pigments in Yeasts, *Jour. Bact.,* **67**(6): 708–713 (1954).

aff, H. J., and E. M. Mrak: Sporulation in Yeasts, Part I, *Wallerstein Labs. Communs.,* **11**(35): 261–279 (1948); Part II, *Wallerstein Labs. Communs.,* **12**(36): 29–44 (1949).

——, ———, and O. B. Williams: Yeasts Isolated from Shrimp, *Mycologia,* **44**(4): 431–451 (1952).

escott, S. C., and C. G. Dunn: "Industrial Microbiology," 2d ed., McGraw-Hill Book Company, Inc., New York, 1949.

octor, B. E.: *Proc. Am. Acad. Arts Sci.,* **69**: 315 (1934).

ke, M.: Biochemical Aspects of Fermentation in Industry, *Jour. Appl. Bact.,* **18**: 187–195 (1955).

chards, O. W.: *Jour. Am. Chem. Soc.,* **47**: 1671 (1925).

——: The Stimulation of Yeast Growth by Thallium, a "Bios" Impurity of Asparagine, *Jour. Biol. Chem.,* **96**: 405–418 (1932).

—— and M. C. Troutman: Spectroscopic Analysis of the Mineral Content of Yeast Grown on Synthetic and Natural Media, *Jour. Bact.,* **39**(6): 739–746 (1940).

elofsen, P. A., and I. Hoette: Chitin in the Cell Wall of Yeasts, *Antonie van Leeuwenhoek Jour. Microbiol. Serol.,* **17**: 24–43 (1951).

man, W., M. Pike, W. J. Nickerson, A. S. Schultz, A. S. W. Thorne, H. Schanderl, K. Arima, and A. C. Thaysen: "Yeasts," Dr. W. Junk, Publishers, The Hague, 1956.

hade, A. L.: Growth Substances in Yeast, *Wallerstein Labs. Communs.,* **2**(4): 36–40 (1938).

——: The Yeasts, *Wallerstein Labs. Communs.,* **4**(13): 192–193 (1941).

oen, M.: "The Problem of Fermentation," Chapman & Hall, Ltd., London, 1928.

——: Faits nouveaux et nouvelles hypothèses dans la chimie des fermentations, *Ann. fermentations,* **4**: 193–219, 257–283, 321–345 (1938). (A review.)

ultz, A. S., L. Atkin, and C. N. Frey: Thiamine, Pyrimidine and Thiazole as Bios Factors, *Jour. Am. Chem. Soc.,* **60**: 490 (1938).

frine, M., H. J. Phaff, and A. L. Demain: Determination of Carbon Assimilation Patterns of Yeasts by Replica Plating, *Jour. Bact.,* **68**(1): 28–35 (1954).

nner, C. E., C. W. Emmons, and H. M. Tsuchiya: "*Henrici's* Molds, Yeasts, and Actinomycetes," John Wiley & Sons, Inc., New York, 1947.

rne, R. S. W.: *Jour. Inst. Brewing,* **43**: 288 (1937).

——: The Nitrogen Nutrition of Yeast, *Wallerstein Labs. Communs.,* **9**(27): 97–114 (1946).

maine, J. H., and J. J. Miller: Effect of Six Vitamins on Ascospore Formation by an Isolate of Bakers' Yeast, *Botan. Gaz.,* **115**: 311–322 (1954).

—— and ———: Effect of Yeast Extract, Peptone, and Certain Nitrogen Compounds on Sporulation of *Saccharomyces cerevisiae. Mycopathol. et Mycol. Appl.,* **7** (fasc. 3–4): 241–250 (1956).

der Walt, J. P.: On the Yeast *Candida pulcherrima,* doctoral dissertation, Delft, Holland, 1952.

lker, T. K., and A. E. Wiles: Asporogenous Film-forming Yeasts Occurring as Contaminants of Brewery Yeasts and Beers, *Jour. Inst. Brewing,* **53**(2): 140–145 (1952).

lerstein, J. S.: New Facts about Growth Substances, *Wallerstein Labs. Communs.,* **4**(11): 14–19 (1941).

— and A. L. Schade: Some Considerations on the Nature of Yeast. I. The

Structure and Functions of the Cell, *Wallerstein Labs. Communs.,* **3**(9): 91–1 (1940); II. Their Origin and Relation to Other Organisms, *Wallerstein Lab Communs.,* **3**(10): 182–198 (1940).

White, J.: The Technique of Yeast Production, *Jour. Appl. Bact.,* **18**: 168–17 (1955).

Wickerham, L. J.: A Simple Technique for the Detection of Melibiose-fermentin Yeast, *Jour. Bact.,* **46**(6): 501–505 (1943).

———: A Critical Evaluation of the Nitrogen Assimilation Tests Commonly Use in the Classification of Yeasts, *Jour. Bact.,* **52**(3): 293–301 (1946).

——— and A. A. Andreasen: The Lyophil Process. Its Use in the Preservation o Yeast, *Wallerstein Labs. Communs.,* **5**(16): 165–168 (1942).

——— and E. Duprat: A Remarkable Fission Yeast, *Schizosaccharomyces versatili* Nov. Sp., *Jour. Bact.,* **50**(5): 597–607 (1945).

——— and M. H. Flickinger: Viability of Yeasts Preserved Two Years by the Lyophi Process, *Brewers Dig.,* **21**(4) (April, 1946).

———, ———, and K. A. Burton: A Modification of Henrici's Vegetable-juice Sporulation Medium for Yeasts, *Jour. Bact.,* **52**(5): 611–612 (1946).

———, L. B. Lockwood, O. G. Pettijohn, and G. E. Ward: Starch Hydrolysis an Fermentation by the Yeast *Endomycopsis fibuliger, Jour. Bact.,* **48**(4): 413–42 (1944).

Wiles, A. E.: The Wild Yeast Problem as a Facet of Brewing Technology, *Wallerstein Labs. Communs.,* **17**(59): 259–281 (1954).

### Some References concerning the Identification and Classification of Yeasts

Bedford, C. L.: Genus *Hansenula* Sydow. Morphological and Physiological Studies doctoral thesis, University of California, Berkeley, Calif., 1941.

———: A Taxonomic Study of the Genus *Hansenula, Mycologia,* **34**: 628–649 (1942)

Custers, M. Th. J.: Onderzoekingen over het gistgeslacht *Brettanomyces,* thesis Technische Hoogeschool, Delft, Holland, 1940, translated by H. J. Phaff and H. C. Douglas, University of California, Berkeley, Calif.

Diddens, H. A., und J. Lodder: "Die anaskosporogenen Hefen," Zweite Hälfte, N. V Noord-Hollandsche Uitgevers Maatschappij, Amsterdam, 1942.

Guilliermond, A.: "Clef dichotomique pour la détermination des levures," Librairie de François, Paris, 1928.

———: "La sexualité, le cycle de développement, la phylogénie, et la classification des levures d'après les travaux récents," Masson et Cie, Paris, 1937.

Henrici, A. T.: The Yeasts: Genetics, Cytology, Variation, Classification, and Identification, *Bact. Revs.,* **5**: 97–179 (1941).

Ingram, M.: "An Introduction to the Biology of Yeasts," Pitman Publishing Corporation, New York, 1955.

Lodder, J.: Die anaskosporogenen Hefen, Erste Hälfte, *Verhandel. Koninkl. Ned. Akad. Wetenschap. Afdel. Natuurk.,* Sec. **II**, **32**: 1–256 (1934).

——— and N. J. W. Kreger-van Rij: "The Yeasts, a Taxonomic Study," North Holland Publishing Co., Amsterdam, 1952.

Phaff, H. J.: A Proposal for Amendment of the Diagnosis of the Genus *Pichia* Hansen, *Antonie van Leeuwenhoek Jour. Microbiol. Serol.,* **22**: 113–116 (1956).

——— and E. P. Knapp: The Taxonomy of Yeasts Found in Exudates of Certain Trees and Other Natural Breeding Sites of Some Species of *Drosophila, Antonie van Leeuwenhoek Jour. Microbiol. Serol.,* **22**: 117–130 (1956).

———, M. W. Miller, and M. Shifrine: The Taxonomy of Yeasts Isolated from *Drosophila* in the Yosemite Region of California, *Antonie van Leeuwenhoek Jour. Microbiol. Serol.,* **22**: 145–161 (1956).

Schultz, A. S., and L. Atkin: The Utility of Bios Response in Yeast Classification and Nomenclature, *Arch. Biochem.,* **14**(3): 36 (1947).

Skinner, C. E.: The Yeast-like Fungi: *Candida* and *Brettanomyces, Bact. Revs.,* **11**(4): 227–274 (1947). (Article contains 303 references.)

——— and R. Bouthilet: Melibiose Broth for Classifying Yeasts, *Jour. Bact.,* **53**(1): 37 (1947).

elling-Dekker, N. M.: Die Sporogenen Hefen, *Verhandel. Koninkl. Ned. Akad. Wetenschap. Afdel. Natuurk.*, Sec. II, **28**: 1–547 (1931).

ickerham, L. J.: A Simple Technique for the Detection of Melibiose-fermenting Yeast, *Jour. Bact.*, **46**: 501–505 (1943).

——: A Critical Evaluation of the Nitrogen Assimilation Tests Commonly Used in the Classification of Yeasts, *Jour. Bact.*, **52**(3): 293–301 (1946).

——: Taxonomy of Yeasts, *U.S. Dept. Agr., Tech. Bull.* 1029, May, 1951.

——: Recent Advances in the Taxonomy of Yeasts, *Ann. Rev. Microbiol.*, **6**: 317–332 (1952).

iles, A. E.: Wild Yeasts: Their Characterization and Classification, *Jour. Appl. Bact.*, **18**: 204–211 (1955).

## Some References concerning Yeast Genetics

owell, R. R.: The Hybridization of Yeasts, *Jour. Appl. Bact.*, **18**: 149–160 (1955).

arlan, P.: Fundamentals of Genetics, *Wallerstein Labs. Communs.*, **9**(26): 5–27 (1946).

enrici, A. T.: The Yeasts, Genetics, Cytology, Variation, Classification and Identification, *Bact. Revs.*, **5**: 97–179 (1941).

ngram, M.: "An Introduction to the Biology of Yeasts," Pitman Publishing Corporation, New York, 1955.

ruis, K., and J. Satava: O vyvoji a klieeni spor jakoz; sexua lite krasinek, V. Praze, 1918.

ndegren, C. C.: The Improvement of Industrial Yeasts by Selection and Hybridization, *Wallerstein Labs. Communs.*, **7**(22): 153–168 (1944).

——: Yeast Genetics, Life Cycles, Cytology, Hybridization, Vitamin Synthesis, and Adaptive Enzymes, *Bact. Revs.*, **9**: 111–170 (1945).

——: Breeding Yeasts for Their New Role in Nutrition, *Missouri Botan. Garden Bull.*, **34**: 37–43 (1946).

——: "The Yeast Cell, Its Genetics and Cytology," Educational Publishers, Inc., St. Louis, 1949.

atava, J.: Oredukovaných formăch krasinek, V. Praze, 1918.

——: Les formes sexuelles et asexuelles des levures et leur pouvoir fermentatif—III$^e$ Congrès international technique et chimique des industries agricoles, Paris, 1918.

iegelman, S., and C. C. Lindegren: The Relation of Sporulation and the Range of Variation of the Haplophase to Population Adaptation, *Jour. Bact.*, **49**: 257–269 (1945).

——, ———, and G. Lindegren: Maintenance and Increase of a Genetic Character by a Substrate—Cytoplasmic Interaction in the Absence of the Specific Gene, *Proc. Nat. Acad. Sci.*, **31**: 95–102 (1945).

ickerham, L. J., and K. A. Burton: Occurrence of Yeast Mating Types in Nature, *Jour. Bact.*, **63**: 449–451 (1952).

—— and ———: Hybridization Studies Involving *Saccharomyces lactis* and *Zygosaccharomyces ashbyi*, *Jour. Bact.*, **71**: 290–295 (1956).

—— and ———: Hybridization Studies Involving *Saccharomyces fragilis* and *Zygosaccharomyces dobzhanskii*, *Jour. Bact.*, **71**: 296–302 (1956).

inge, Ö.: On Haplophase and Diplophase in Some Saccharomycetes, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **21**(4): 77–111 (1935).

—— and O. Laustsen: On Two Types of Spore Germination, and on Genetic Segregations in *Saccharomyces*, Demonstrated through Single-spore Cultures, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22**(6): 99–116 (1937).

—— and ———: Artificial Species Hybridization in Yeast, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22**(13): 235–244 (1938).

—— and ———: On 14 New Yeast Types, Produced by Hybridization, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22**: 337–352 (1939).

—— and ———: *Saccharomycodes ludwigii* Hansen, a Balanced Heterozygote, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22**: 357–370 (1939).

—— and ———: On a Cytoplasmic Effect of Inbreeding in Homozygous Yeast, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **23**: 17–39 (1940).

## Some References concerning Yeast Flocculation

Barendrecht, H. P.: *Zentr. Bakt. Parasitenk.*, **7**: 623 (1901).

Beijerinck, M. W.: *Zentr. Bakt. Parasitenk.*, II, **20**: 641 (1908).

Brohult, S.: General Report. Yeast Flocculation and Primary Fermentation, “Eu
pean Brewery Convention—Proceedings of the Congress Brighton 1951,” Elsev
Publishing Company, Amsterdam, 1951, pp. 3–9.

———, W. Lindquist, and E. Sandegren: High Molecular Substances in Yea
“European Brewery Convention—Proceedings of the Congress Brighton 195
Elsevier Publishing Company, Amsterdam, 1951, pp. 188–194.

Burns, J. A.: Yeast Flocculation, *Jour. Inst. Brewing,* **43**: 31–43 (1937).

DeClerck, J.: La conduite de la fermentation, *Extrait du Bull. assoc. anciens étu*
*brass. univ. Louvain,* **48**(1): 9–19 (1952).

——— et G. Van Roey: Courbe de fermentation et flocculation de la Levure, *Extr*
*du Bull. assoc. anciens étud. brass. univ. Louvain,* **48**(1): 1–7 (1952).

De Hemptinne, Y.: *Fermentatio,* No. 4, 1949, p. 58.

Dunn, C. G.: Yeast Flocculation, *Am. Brewer,* December, 1955, pp. 42–46.

Geys, K.: *Jour. Inst. Brewing,* **28**: 479 (1927).

Gilliland, R. B.: The Flocculation Characteristics of Brewing Yeasts during Ferment
tion, “European Brewery Convention—Proceedings of the Congress Bright
1951,” Elsevier Publishing Company, Amsterdam, 1951, pp. 35–58.

Hartong, B. D.: De Vlokking van de Gist in de Brouwerij, *Overdruk uit Brouwe*
*en Mouterij,* No. 9–10, 1951, pp. 143–148.

———: Flocculation of Yeast in the Brewery, “European Brewery Convention
Proceedings of the Congress Brighton 1951,” Elsevier Publishing Compan
Amsterdam, 1951, pp. 110–119.

Heilbron, I.: Brewing in Relation to Natural Science, *Proc. Roy. Soc. (London*
**B**(911), **143**: 178–191 (1955).

Helm, E., B. Nøhr, and R. S. W. Thorne: The Measurement of Yeast Flocculen
and Its Significance in Brewing, *Wallerstein Labs. Communs.,* **16**(55): 315–3
(1953).

Hind, H. L.: “Brewing Science and Practice,” Vol. II, “Brewing Processes,” Joh
Wiley & Sons, Inc., New York, 1940, pp. 778–782, 792, 793, 808, 816, 817.

Hopkins, R. H., W. O. Kermack, and W. T. Spragg: *Biochem. Jour.,* **25**: 829, 18
(1931).

Jansen, H. E., and F. Mendlik: A Study on Yeast Flocculation, “European Brewe
Convention—Proceedings of the Congress Brighton 1951,” Elsevier Publishin
Company, Amsterdam, 1951, pp. 59–83.

——— and ———: A Study on Yeast Flocculation, II, “European Brewery Conve
tion—Proceedings of the Congress Nice 1953,” Elsevier Publishing Compan
Amsterdam, 1953, pp. 143–154.

Kijima, M.: *Wallerstein Labs. Communs.,* **58**: 227 (1954).

Koch, R.: *Wochschr. Brau.,* **45**: 175, 187, 201 (1928).

Lund, A.: Flocculation as a Racial Character in Bottom Fermentation Yeast, “Euro
pean Brewery Convention—Proceedings of the Congress Brighton 1951,” Elsevie
Publishing Company, Amsterdam, 1951, pp. 10–20.

Malkow, A.: *Zentr. Bakt. Parasitenk.,* **90**: 212 (1934).

Nielsen, N.: *Compt. rend. trav. lab. Carlsberg, Sér. physiol.,* **22**: 61 (1937).

Pomper, S., and D. W. McKee: *Science,* **117**: 62 (1953).

Rohrer, E.: Yeast Flocculation, *Brewers Dig.,* **25**(4): 54, 55 (1950).

St. Johnston, J. H.: The Termination of Primary Fermentation. I. Experiment
Data Obtained from Flocculation and Fermentation Tests, *Wallerstein Lab*
*Communs.,* **16**(52): 39–70 (1953).

———: The Termination of Primary Fermentation, II. Experimental Data on th
Rising of Yeast in Top Fermentation, *Wallerstein Labs. Communs.,* **16**(53
119–132 (1953).

Schönfeld, A., and H. Krumhaar: *Wochschr. Brau.,* **35**: 302, 342 (1918).

Silbereisen, K.: *Wochschr. Brau.,* **55**: 20, 153, 171 (1938).

piegelman, S., and O. E. Landman: Genetics of Microorganisms, *Ann. Rev. Microbiol.*, **8**: 211 (1954).

tockhausen, F.: *Wochschr. Brau.*, **44**: 121, 133 (1927).

horne, R. S. W.: Some Aspects of Yeast Flocculence, "European Brewery Convention—Proceedings of the Congress Brighton 1951," Elsevier Publishing Company, Amsterdam, 1951, pp. 21–34.

——: The Problem of Flocculence in Brewery Yeasts, *Wallerstein Labs. Communs.*, **15**(50): 201–211 (1952).

——: Fermentation Velocity of Yeasts, I. Measurement of Fermentation Velocity and the Effects of Experimental Conditions, *Jour. Inst. Brewing*, **60**(3) (**51**, New Series): 227–237 (1954).

——: Fermentation Velocity of Yeasts, II. A Study of the Fermentation Velocities of a Series of Bottom and Top Fermentation Brewery Yeasts, *Jour. Inst. Brewing*, **60**(3) (**51**, New Series): 238–248 (1954).

nderkofler, L. A., and R. J. Hickey: "Industrial Fermentations," Chemical Publishing Company, Inc., New York, 1954, Vol. I, p. 300.

hite, J.: "Yeast Technology," John Wiley & Sons, Inc., New York, 1954, pp. 338–339.

atsushiro, T.: Correlation of Flocculation with Malt Quality, Abstract, *Wallerstein Labs. Communs.*, **17**(57): 150 (1954).

## CHAPTER 3

# THE PROPAGATION OF YEAST

### BAKERS' YEAST

**Development of Industry.** The baking of bread has been practiced for centuries. Egyptian, Jewish, Greek, and Roman histories reveal many interesting facts concerning baking, brewing, and wine making. The Bible suggests a method for preparing leavened bread.

Mason, an Englishman, is said to have prepared the first compressed yeast in 1792 (Frey, 1930). No hops were used in its manufacture. Shortly thereafter, compressed yeast was known in Holland and in Germany, as well as in England. Indeed, there is some evidence that compressed yeast was made in Holland as early as 1781.

Leavened bread was prepared for many years by mixing a portion of leftover dough with the main batch and permitting the mix to stand for a while to undergo fermentation. A more modern practice was to mix residual yeast, from the brewery or distillery, with the dough. Such yeast often possessed a bitter taste owing to the use of hops in malt-beverage manufacture.

**The Vienna Process.** Some (Frey et al., 1936) consider that the compressed-yeast industry started with the development of the Vienna process, a modified brewing operation, about 1860. In this process, kiln-dried malt and corn were ground, mixed with water, and mashed (see the chapter on Brewing). The unfiltered mash was pitched and permitted to ferment. Machines separated the portions of grain from the yeast by a process of sifting. Yields of 10 to 14 per cent on the basis of the grain were obtained along with a considerable amount (about 30 per cent) of ethyl alcohol.

Since compressed-yeast manufacturers are interested in the yield of yeast and not the alcohol, improvements in this method were made from time to time. A process for aerating mashes for yeast production was developed by Marquardt in 1879. Yields of yeast were materially increased, while those of alcohol were decreased. The filtration of the wort, the use of very dilute worts, carefully regulated aeration, and fairly low temperatures were later modifications that still further improved the process.

Many of the modern methods of compressed-yeast manufacture are protected by patents.

Soon after the commencement of World War I, research was carried out in Germany which led to the development of some of the modern methods of manufacture. The high cost and scarcity of grains had much to do with the instigation of this research.

For a more extended discussion of the development of the yeast industry, the reader is referred to the report of Frey (1957).

## SOME GENERAL CONSIDERATIONS CONCERNING THE PRODUCTION OF YEAST

**Yeast.** Special strains of *Saccharomyces cerevisiae* are used in the baking industry. These strains must possess certain properties, such as biochemical stability (uniform and constant characteristics), ability to ferment the flour dough vigorously (excellent dough-raising power), ability to disperse readily in water, good keeping qualities (ability to resist autolysis) and good appearance for a reasonable period at normal handling temperatures, and ability to reproduce well and in good yield in the propagation medium. Reproduction by budding is shown in Fig. 10.

**Nutrient Materials.** In order to obtain maximum yields of yeast, the media used in propagation must contain the required nutrients, organic and inorganic, in adequate amounts.

The carbon is generally supplied by the sugars contained in the molasses, cane and beet, in the saccharified grains, or in another source. Carbon may also be supplied in somewhat limited quantity by such nonsugar sources as amino acids, lactic acid, ethyl alcohol, and other substances.

Nitrogen may be supplied as aqueous ammonia; ammonium salts; soluble protein derivatives, such as peptones, peptides, and amino acids; or urea. Nitrates and nitrites are not acceptable. Corn-steep liquor may serve as a source of organic nitrogen when available. It has been reported by Menzinsky (1950) that molasses "with a lower sugar content and a higher organic nitrogen content gives a better yield than a material with a higher sugar and lower nitrogen."

Phosphorus is an essential nutrient and may be supplied as diammonium phosphate, disodium phosphate, calcium acid phosphate, phosphoric acid, or in other form.

Magnesium, in the form of magnesium sulfate heptahydrate, may be added to the wort, if it is found to be deficient in amount, as a result of laboratory analysis of the raw material.

Bakers' yeast also requires certain vitamins or bios factors for maximum yields. White and Munns (1950) found that one strain of yeast required 0.29 p.p.m. of available biotin, 50 p.p.m. of available pantothenate, and 1,200 p.p.m. of available inositol for optimum yield from beet molasses containing 50 per cent of fermentable sugar. Beet molasses was found to be an excellent source of pantothenate and inositol but was deficient in biotin. Blackstrap, refiners' cane, and high-test cane molasses were excellent sources of biotin but were poorer sources of pantothenate and inositol than beet molasses. Actually, refiners' cane molasses and high-test cane molasses were deficient in respect to pantothenate. On account of its deficiency in pantothenate and inositol, high-test cane molasses was considered to be of limited value for yeast manufacture.

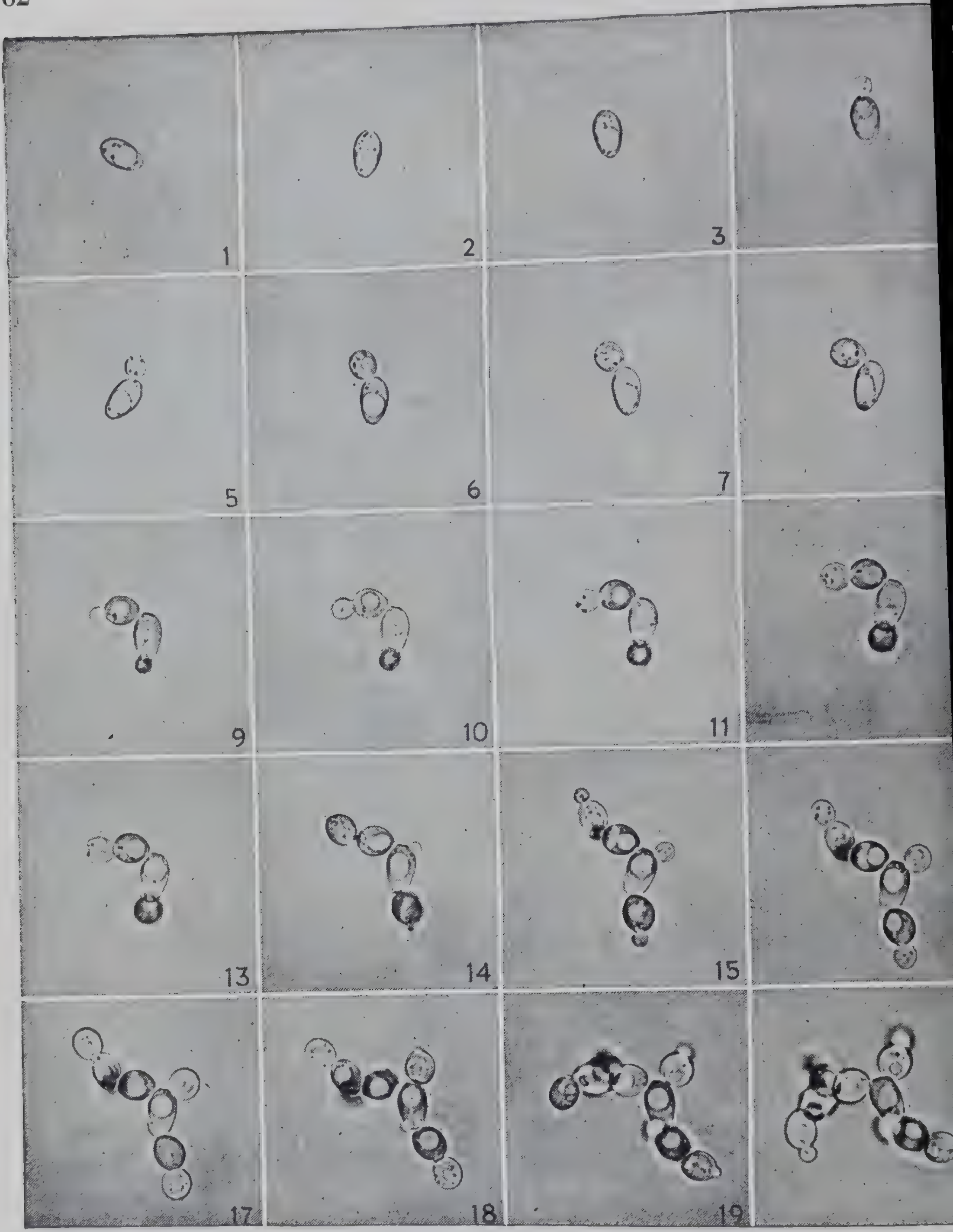


FIG. 10. Budding yeast cells. Magnified 1,500 times. Pictures taken at 15-min. i
vals. (*Courtesy of C. N. Frey, Fleischmann Laboratories.*)

Menzinsky (1950) also found that the yield of yeast from molasses
highly dependent upon the biotin content of the medium. Vitamin
(aneurine) and inositol exhibited some effect on yeast growth.
pantothenic acid and pyridoxine did not affect the yield under the co
tions used.

*Concentration of Sugar.* The concentration of sugar is maintained
low level in the fermentation (propagation) tank in order to favor
utilization for the manufacture of yeast cells rather than for the pro

tion of ethyl alcohol. The usual concentrations in the fermentor vary from about 0.5 to 1.5 per cent, depending on the process. The concentration of sugar in the wort is maintained at this low level throughout the period of production regardless of whether the latter is on a semicontinuous or continuous basis. This is accomplished by admitting a concentrated wort at a predetermined constant or logarithmically increasing slow rate.

*Use of Lactic Acid Bacteria.* Frequently in the manufacture of yeast from cereal grain, the filtered mash may be inoculated with a culture of *Lactobacillus delbrueckii* and maintained for a while at a temperature of 50 to 52°C. to favor the production of lactic acid. Yeasts grow well in the media containing lactic acid, while contamination, especially with butyric acid organisms, is reduced. The presence of lactic acid in the yeast cake inhibits the development of bacteria.

**pH.** The pH of the propagation mash should be maintained rather uniformly throughout the process. Different yeast manufacturers use different values. However, values lying between 3.5 and 4.5 appear to be satisfactory. The English use rather low pH values, while in this country the values may range between 4 and 5. Menzinsky (1950) found that varying the pH within the range of 4 to 6 had no influence on the yield. Low pH is helpful in restricting the development of many bacterial species. However, the color of the yeast may suffer if the pH is too low.

By using a proper combination of aqua ammonia and ammonium sulfate, it may be possible to regulate the pH to a certain degree. Sulfuric acid, ammonia, sodium carbonate, or sodium bicarbonate may be used to adjust the pH when required.

**Aeration.** Adequate aeration is one of the most important factors in yeast production. Large amounts are used.

The surface area of a given volume of bubbles of air in a liquid increases as the sizes of the bubbles become smaller. There is thus a larger contacting surface with small-sized bubbles than with large ones and a longer period of contact with the medium or wort. A smaller quantity of air is required when the bubbles used are of small size, but it costs more to produce very fine bubbles. Thus, the yeast manufacturer must select a desirable course, in which efficiency of aeration is high but not too costly. The bubbles used commercially vary from 0.0001 to 1 in. in diameter, according to de Becze and Liebmann (1944) who have thoroughly discussed the subject of aeration. Aeration pipes with $^{1}/_{64}$- to $^{1}/_{32}$-in. openings are often used with good success. Aerators of the porous varieties may be used for fine aeration but tend to clog more easily and require greater air pressure.

Air is introduced near the bottom of the fermentation tank, and the aeration network should be of such nature that there is uniform distribution of air over the entire cross section of the tank.

The air requirements for a wide and shallow fermentor tank will be much different from those of a narrow, tall tank, for the longer a bubble of air is in contact with the medium, the more effective it is. Based on the yeast produced, a considerably larger amount of air is required for a small fermentor than for a large one.

de Becze and Liebmann reported that the correct evaluation of air requirements must be based on information available concerning the height of the wort at any time (obtained by reference to a chart or graph); the concentration of yeast at any time (by graph); the yield of yeast, per unit of raw material (by graph); and the ratio of the amount of new yeast to seed yeast.

Amounts of air required for yeast production, as reported by de Becze and Liebmann (1944), varied from 275 to 530 cu. ft. per lb. of new yeast of 30 per cent solids content. White (1954) reported an air requirement of 7,000 c.f.m. during the eleventh hour for a vat in which 8,400 lb. of yeast (net) of 30 per cent solids content was produced.

Air requirements are generally reported in terms of cubic feet per (1) gallon of wort, (2) square foot of wort or fermentor bottom surface, (3) pound of yeast in 100 gal. of wort, (4) pound of yeast produced, (5) ton of total molasses, or (6) bushel of grain mashed; or milliliters, liters, or cubic meters per unit volume of medium, etc.

Aerators are of many types. For example, they may be plates, tubes, candles, spargers, or false bottoms. They may be of the fixed or movable type. They may be constructed of noncorrosive metal, ceramic substances, hard or soft rubber, porous carbon, sintered glass, canvas, or other substances.

*Function of Oxygen.* The exact function of oxygen in the growth of yeast is not known. Very likely its action may be due to several factors: inhibition of fermentation and increase of respiration, agitation of the medium, removal of toxic end products, and stimulation of vegetative growth.

*Temperature.* The temperature is generally started at 25 or 26°C. It is usually held fairly close to this level during the early part of the fermentation and gradually permitted to rise to 30°C. toward the end of the fermentation. Cooling and heating coils are an essential part of the fermentation-tank equipment.

*Preserving the Yeast.* The use of a low temperature is necessary for the storage of compressed yeast. Molds and bacteria cause the cake to deteriorate rapidly at room temperature, and autolysis is likely to take place.

Various methods for improving the keeping qualities of yeasts have been proposed. For example, it has been suggested that washing the yeast for 1 hr. with a solution of ethyl, propyl, isopropyl, butyl, or amyl alcohol before pressing improves the keeping quality. The use of colloids—pectin, agar, gelatin, gum tragacanth, dextrin, or algae extract—to remove the water from yeast, followed by drying to a definite moisture content at a temperature that will not injure the yeast, has also been proposed.

**Building up the Yeast Culture.** In the plant, the practice is to start with a single cell and to build it up by means of successive transfers until a sufficient mass of cells has been accumulated to seed the main mash or wort. This is done with great care to prevent contamination with microorganisms. The micromanipulator is an excellent tool for isolating pure cultures of yeast. After the single cell has been isolated, it is grown on a slant or in broth in a culture tube. A flask containing a sterilized, well-balanced molasses mash or grain wort, with a content of about 6 per cent of sugar, is inoculated with a loopful of the yeast. After incubation for 12 or more hr. at 25 to 30°C. under anaerobic conditions, the contents of the flask are used to seed a flask containing a larger volume of a mash of similar composition. This process may be repeated with progressively larger mashes until sufficient seed is available to inoculate tanks situated in the plant proper.

It is desirable to build up the yeast cultures in a special room in the plant. This room should be constructed in such a manner as to provide maximum freedom from contamination. It should be designed to be easily cleaned and sanitized and should be operated under a slight positive pressure with filtered air.

In Table 19 are presented data supplied by White (1949) regarding the building of the culture through five plant stages (F.1. to F.5.). The molasses worts used in stages F.1. to F.3. contain approximately 5 per cent of fermentable sugar. The pH should be 4.2 to 5 (occasionally lower). The worts should be boiled for 1 hr. and cooled before inoculation. The fermentation time for each stage is 12 hr., and the temperature 29 to 32°C. In stages F.4. and F.5. the wort contains about 10 per cent of sugar. Additional stages may be used if desired.

**Seed Yeast.** Both the quality and the quantity of seed yeast are important in obtaining good yields. Usually about 20 per cent of seed yeast, based on weight of the molasses, is used for seeding the commercial run. Menzinsky (1950) found that increased amounts of seed yeast, within certain limits, resulted in higher yields of yeast and that the content of vitamins in the seed yeast was the factor of prime importance in determining its yeast-forming efficiency. Frey advocates the use of seed yeast with a high protein value (50 per cent or greater).

**Flow Diagram.** Figure 11 presents a flow diagram of compressed-yeast manufacture.

**The Molasses-Ammonium Process.** The raw materials for this process are generally a mixture of beet and cane molasses; aqueous ammonia, ammonium sulfate, and ammonium dihydrogen phosphate; disodium phosphate, phosphoric acid, or other source of phosphorus; and, occasionally, magnesium sulfate heptahydrate. The relative amounts of beet and cane molasses employed will depend on their respective availabilities and costs. A 60:40 ratio of the two may produce good yields. Of course, beet molasses may be employed without the addition of cane molasses.

Best yields will be obtained, other factors remaining constant, when the nitrogen, phosphorus, and magnesium requirements are carefully determined. White (1949) added 1.8 per cent of nitrogen, calculated on the weight of beet molasses containing 50 per cent of total sugar (as invert). He added phosphorus, as $P_2O_5$, in the amount of 0.8 per cent on the same basis. The exact requirements of a given mash for a given

Table 19. Details of Yeast Culture Stages *

(F.1. to F.5.)

| Stage | Volume, gal.† | Materials used, lb.† | | | | Yeast present at end of stage, lb. at 30% dry matter | Temperature, °F. | Length of fermentation, hr. | Other details |
|---|---|---|---|---|---|---|---|---|---|
| | | Molasses (beet) | Nitrogen | Phosphate | Other materials | | | | |
| F.1. | 10 | 10 | 0.06 | 0.05 | | 2 | 85 | 12 | Aerated for a few minutes each hour. |
| F.2. | 40 | 40 | 0.24 | 0.2 | | 8 | 85 | 12 | Aerated for a few minutes each hour. |
| F.3. | 200 | 200 | 1.2 | 1.0 | | 40 | 85 | 12 | Aerated for a few minutes each hour. |
| F.4. | 750 | 800 | 5.0 | 4.5 | 1 lb. MgO | 200 | 85 | 12 | Very gentle aeration at about 100 c.f.m. |
| F.5. | 3,000 | 3,200 | 20 | 18 | 3 lb. MgO | 1,000 | 85 | 12 | Aeration throughout at 1,000 c.f.m. |

Notes: Laboratory culture yields about 0.5 lb. of yeast for seeding F.1. Nitrogen may be introduced as ammonium sulfate, in which case the amount of ammonium sulfate = nitrogen × 4.71. Phosphate may be added as $Na_2HPO_4 \cdot 12H_2O = P_2O_5 \times 5.04$. Magnesium may be added as sulfate ($MgO \times 6.11 = MgSO_4 \cdot 7H_2O$). Grain-wort system: Use equivalent quantities of fermentable sugars as shown above and add about 2 per cent of diammonium phosphate to the wort. Beet-molasses weights based on total sugar (as invert) content of 50 per cent.

* J. White, *Am. Brewer*, August, 1949, p. 28.

† English gallon = 4,546 ml.; English pound = 453.6 g.

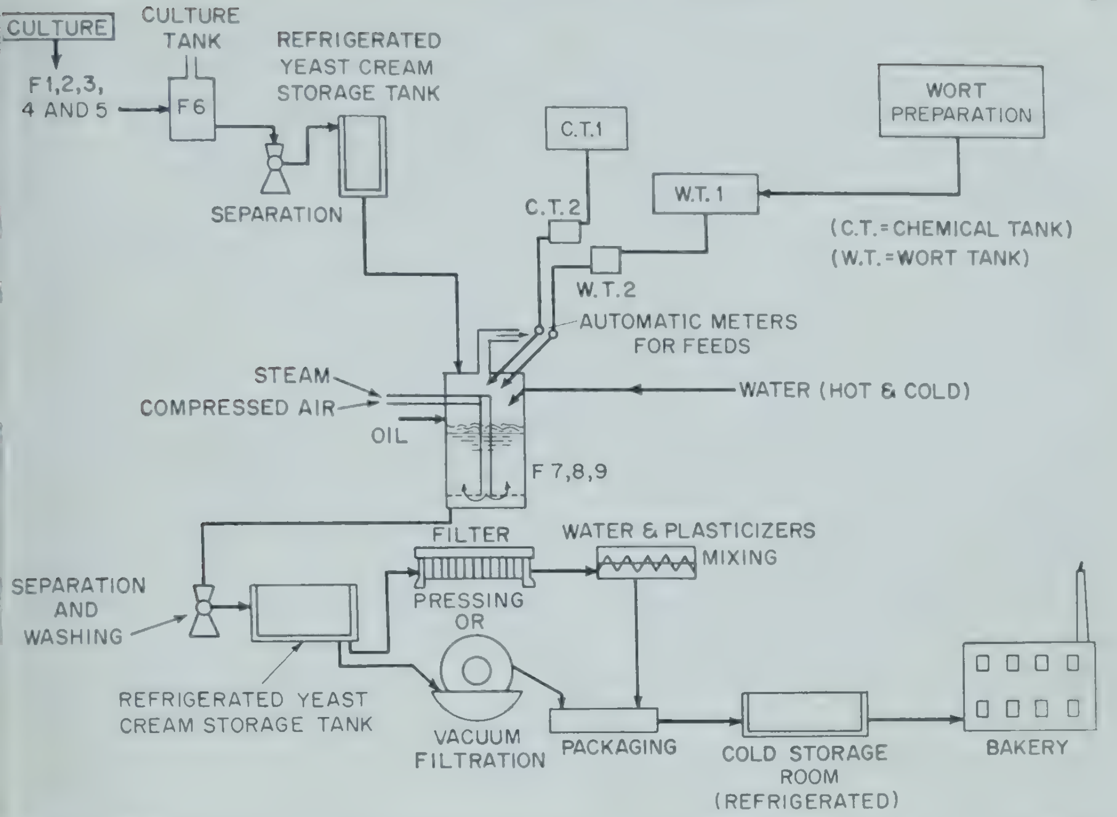

CULTURE
F1,2,3, 4 AND 5
CULTURE TANK
F6
SEPARATION
REFRIGERATED YEAST CREAM STORAGE TANK
C.T.1
C.T.2
W.T.1
W.T.2
WORT PREPARATION
(C.T.=CHEMICAL TANK)
(W.T.=WORT TANK)
AUTOMATIC METERS FOR FEEDS
STEAM
COMPRESSED AIR
OIL
WATER (HOT & COLD)
F 7,8,9
FILTER
WATER & PLASTICIZERS
MIXING
PRESSING OR
SEPARATION AND WASHING
REFRIGERATED YEAST CREAM STORAGE TANK
VACUUM FILTRATION
PACKAGING
COLD STORAGE ROOM (REFRIGERATED)
BAKERY

Fig. 11. Flow diagram of compressed-yeast manufacture. (*Courtesy of John White, Am. Brewer, August,* 1949, *p.* 29.)

ingredient may be calculated only after the molasses has been chemically analyzed.

Molasses is heat-treated, clarified, and diluted before its use. It is usually adjusted to a pH of 4.5 to 5 and then heated nearly to its boiling point. Where corn-steep liquor is used, this is added to the molasses before heat-treatment. Heating destroys a large percentage of the microorganisms present and aids in the clarification process which follows. Heating may be accomplished through the use of heat exchangers, by the circulation of steam through the coils or the jacket of a tank, or by the direct injection of steam. Clarification is accomplished by the use of centrifuges, filters, or decantation following sedimentation. The clarified molasses is diluted with water of high purity, and this constitutes the concentrated feed wort.

A number of actions are initiated and take place in the propagator, which is carefully cleaned and sanitized between runs. Pure water is introduced into the propagator. The phosphate, or a part of it, may be dissolved in a portion of this start water. Some of the ammonium sulfate and all of the magnesium required may also be mixed with some of the tart water in the propagator. The strong wort is added in small amounts o yield a very low sugar concentration, preferably a fraction of a per ent. The total volume of liquid at this time may amount to 70 per cent or more of the total final volume. The seed yeast is added, and aeration is commenced. The strong wort and the chemicals (aqua ammonium and other chemicals not introduced at the start) are fed exponentially to the propagator over a period of 8 to 12 hr. The rate of aeration is increase as the amount of yeast grows. An additional hour is allowed for maturation of the yeast cells. No wort or chemicals are supplied during thi period, and the amount of aeration is greatly reduced.

During the propagation, the temperature, pH, and foam formation ar carefully controlled. The temperature should not be permitted to ris above 30°C. The pH and foam formation are controlled manually o through the use of automatic devices.

The time of propagation is determined by the nature of the substrat the rate of feed, the quality and quantity of the seed yeast, the temperature, the aeration and/or agitation, and other factors.

After propagation the yeast may be cooled rapidly, separated from th spent wort, and washed several times. Separation is performed by centrifuges which have capacities for several thousand gallons per hour. Th yeast cream is mixed with potable water in a wash tank, while the spen liquor is discharged into the sewer. The yeast suspension is separate again into yeast cream and wash water. This operation is repeated a desired, until a clean product is obtained. The washed yeast goes to filte presses, usually of the plate-and-frame type, and emerges as a solid. Th yeast is blended and mixed with a plasticizer. Finally, it is extruded i block form, cut, wrapped, packed in cartons, and placed in refrigerate storage.

Table 20 lists some of the control tests which are carried out in variou yeast plants.

*A Procedure Employed in Germany.* Bakers' yeast was produced i one German plant by the following procedure:[1] Beet molasses is dilute

TABLE 20. CONTROL TESTS USED IN YEAST MANUFACTURING PLANT

| Raw materials | Process control | Finished yeast |
|---|---|---|
| Nitrogen (Kjeldahl) | pH | Nitrogen (Kjeldahl) |
| Amino nitrogen | Amino nitrogen (Formol titration) | Phosphorus |
| Amide nitrogen | Other | Solids content |
| Sugars (total) | | Dispersion in water |
| Reducing sugars (unfermentable) | | Color |
| Total solids | | Consistency |
| Minerals: | | Package weight |
| Phosphorus | | Storage tests: |
| Magnesium | | Keeping qualities |
| Potassium | | Color |
| Others | | Odor |
| Vitamins | | Appearance |
| Color | | Gas power: |
| Other | | Total gas versus time |
| | | Punch tests on dough |
| | | Baking tests |
| | | Other |

---

[1] A. K. Balls, *FIAT Final Report* 277, Oct. 3, 1945.

to 30°Bé and adjusted to pH 4.5. It is then sterilized by heating at 105°C. for 1 hr. The solution is allowed to stand for 4 to 6 hr., during which time suspended solids settle out and the temperature drops to 90°C. It is filtered. Nutrients, in the form of 110 kg. of ammonium sulfate and 250 kg. of superphosphate for each 7,000 kg. of yeast produced, are added. This mixture is held in storage tanks and later fed at a slow predetermined rate to the fermentation tank.

The inoculum is prepared as follows: A strain of *S. cerevisiae*, especially suitable for the type of molasses being used, is grown on a slant. This culture is used to inoculate a 1-liter mash. The size of the inoculum is progressively increased by successive transfer through 50-, 200-, 1,000- and 15,000-liter mashes. Finally a 90,000-liter inoculum is prepared which is used to inoculate six large fermentors of the type described below.

Fermentation (propagation) tanks are constructed of steel and are of 200,000-liter capacity. To start a fermentation, 20,000 liters of water are run into the tank. Sterilized, nutrient molasses is added to the water until the sugar content reaches 1.5 per cent. The inoculum is then added and aeration commenced. The feed of the sterile 30°Bé molasses is then adjusted so that the sugar concentration is kept at 1.5 per cent. The temperature is maintained at 25 to 30°C. The fermentation is over in 10 to 11 hr.

The aerating system consists of perforated copper tubes located at the bottom of the fermentor. For each kilogram of yeast produced, 16 cu. m. of air is supplied. A supply of 4,000 cu. m. of air per hour is necessary for 7,000 kg. of molasses. Crude lanolin is used as an antifoam agent.

The yeast is separated from the spent wort by means of Westphalia entrifuges. The cream so obtained is washed twice, cooled to 8 to 9°C., and pressed in a plate-and-frame press. The resultant cake contains 31 per cent solids. It is diluted with water to 25 per cent solids before being packaged.

**Yeast from Sulfite Liquor by the Heijkenskjöld Method.**[1] Sulfite iquor, mixed with a small quantity of molasses, serves as the source of raw material for the manufacture of yeast by the Heijkenskjöld method.

The first step in this process consists of adjusting the hot sulfite liquor o a pH of 6 to 6.5 with lime, powdered limestone, and sodium carbonate, while the liquor is being aerated in tanks. After the neutralization, the iquor is permitted to stand, in order that the precipitates may settle out. The clear supernatant liquor is decanted from the tanks and cooled to 28 to 30°C. by passage through coolers and is then ready for use in the manufacture of yeast.

The initial mash contains molasses mixed with sulfite liquor to yield sugar concentration of 3 to 5 per cent. Malt sprouts may be added to his mash, if desired, to supply organic nitrogen and other nutrient substances. The mash is now inoculated with the seed yeast, a strain of S. *cerevisiae* ("Rasse 12" of the Gärungsinstitut of Berlin is used in Finland and at the Best plant in Liverpool, Nova Scotia).

Sulfite liquor is fed into the mash after the yeast has commenced to

[1] E. W. Eweson, *Chem. Inds.*, 38: 573 (1936).

develop new cells. Ammonium sulfate and phosphates are added duri the course of the process to furnish nutrient material and to mainta the desired reaction. Large quantities of filtered air are supplied, the removing the carbon dioxide and other gases formed besides carrying c other functions. After the propagation is complete, the yeast is wash repeatedly. The final product is light in color, of high purity, and neutr in taste.

The sulfite liquor used in this process contains approximately 25 of sugar per 1,000 kg. of liquor, and of this amount about 16 kg. is fe mentable. The sugar concentration is thus very low and would not fav the production of alcohol in large amounts.

An average yield of 160 per cent is obtained on the basis of the fe mentable sugar, yields varying from 40 to 55 lb. for each ton of was sulfite liquor.

**Additional Information.** The reader is referred for an extended di cussion of this subject to the works of White (1954, 1955), Walter (195 Frey (1957), Menzinsky (1950), Nordsiek (1951), Keipper (195 Yamada (1956), and others.

## ACTIVE DRY YEAST

Active dry yeast is a product developed for use in areas or places whe stocks of fresh yeast are not available. Active dry yeast does not requi refrigeration, is uniform and stable over required periods of time, convenient to weigh, rehydrates readily, and is economical (Merrit 1957).

The history of the development of the dry-yeast industry has bee comprehensively reviewed by Frey (1957).

A special strain, or strains, of *Saccharomyces cerevisiae* is used in t preparation of active dry yeast (Merritt, 1957). Likewise, a special pro ess is used in growing the yeast, which is then washed and concentrate in the usual manner. The press cake is dispersed in the form of pelle or granules for drying.

Drying of the yeast must be accomplished with great care in order preserve its physiology and prevent injury to the enzymes (Frey, 1957 Under the present conditions of drying, a final moisture content of abo 8 per cent is desired. At a level of 10 per cent moisture yeast does n keep well, and at a content of 5 to 6 per cent moisture there is reduce activity of the yeast during its use. The active dry yeast is packaged containers without any special precautions in respect to the removal air, is packaged after the replacement of the air with nitrogen gas, or packaged under vacuum. The last two procedures are used in case t yeast is to be exported or is destined for long storage.

Active dry yeast should be rehydrated by adding 1 part of it by weig to about 4 parts of water by weight at a temperature of 110°F. Abo 5 min. is required for rehydration.

## FOOD AND FODDER YEAST

"Food yeast," also known as "nutritional yeast," is yeast propagated sentially for food purposes. Yeast produced chiefly to feed animals is lled "fodder yeast." Both are propagated under similar conditions, but od yeast is usually refined and handled under somewhat superior condi- ons. Dried, debittered brewers' yeast, a by-product of the brewing dustry, is used for food and pharmaceutical purposes.

Food yeast is a rich source of protein and vitamins of the B complex. the dry state, it usually contains about 50 per cent of protein, and when ed in the proper proportions with other foods it makes a satisfactory d nutritious supplement to the diet of persons living under conditions ere there may be a shortage of animal proteins or vitamins of the complex.

The manufacture of fodder yeast is of interest to those desiring to nvert waste, surplus, or low-cost carbohydrate materials into products value for feeding cattle, hogs, or other farm animals. The utilization waste sulfite liquors for fodder-yeast production, for example, results the extension of animal foods and at the same time aids in the allevia- n of stream pollution.

Fodder and fodder yeast is being produced in a number of countries roughout the world and is a well-established industry. There are plants the United States, Sweden, the United Kingdom, and other countries. rmany produced and consumed relatively large quantities of food ast during World Wars I and II.

There is an extensive amount of literature available concerning food l fodder yeast. Fink et al. (1937) reported on early research by dner and his associates in Germany and, at a later date, on research cerned with the production, composition, and food value of yeast 38, 1939, 1942). Locke (1946), the Northeastern Wood Utilization uncil (1946), Holderby (1946), Inskeep et al. (1951), Dunn (1952), l others have reviewed the history of the food-yeast process.

**eneral Details of Production.** The following section is concerned h details of a fundamental nature in respect to the propagation of food l fodder yeast.

*Microorganisms Used.* A number of factors must be considered in the ction of a food yeast (Dunn, 1952). The yeast should possess high ritive value, agreeable flavor, biochemical and cultural stability, lity to assimilate a relatively large number of carbon- and nitrogen- taining sources, the ability to grow rapidly in high yield, easy recover- ity, and good appearance.

he yeast commonly used commercially is a strain of *Candida utilis*, wn as "torula yeast" and often called *Torulopsis utilis*. Other yeasts l commercially, or studied extensively in the laboratory, include *rborea*, *C. tropicalis*, *C. pulcherrima*, and *C. reukaufii*; *Hansenula mala* and *H. suaveolens*; *Trichosporon pullulans*; and, of course, *Sac- omyces cerevisiae*. Other yeasts have been studied because of their value as fat-producing microbes and will be referred to later in thi chapter.

*C. utilis* is outstanding in its ability to assimilate a wide variety o carbon and nitrogen sources, including galactose and xylose, and acetic acetoacetic, fumaric, malic, lactic, pyruvic, and succinic acids. It is thu able to utilize hexoses, pentoses, and other constituents found in the aci hydrolyzates from wood or other cellulosic materials. It is particularl desirable on account of its high protein content and its vitamins of th B complex.

*C. utilis* is an asporogenous yeast, the biochemical characteristics o which have been described by Lodder (1934), Sperber (1945), Dun (1952), Lodder and Kreger-van Rij (1952), and others. Fink and Jus (1938, 1939, 1942) have also studied the biochemistry of this yeast.

There are a number of strains of *C. utilis*. One, known as *C. utili major*, produces fairly large cells; another, *C. utilis thermophila*, grow at higher temperatures than many yeasts.

*S. cerevisiae* is frequently the yeast of choice when molasses is the ra material used for propagation purposes. This yeast is unable to assimilate galactose and xylose. It has been studied in detail by Menzinsk (1950) and others.

SIZE OF INOCULUM. Usually the concentration of cells in the inoculu is of such magnitude as to yield 100 million to 200 million cells per ml. i the propagation medium.

*Raw Materials.* In producing food yeast, it is desirable to use materials which are readily available and assimilable and low in cost. On commercial basis, sulfite liquors, molasses, and wood sugars have bee used successfully. However, hydrolyzed grains, citrus press juice, frui juices, protein waste liquors from starch plants, hydrolyzed olive residues and other materials, when properly prepared, may be used.

PRETREATMENT OF RAW MATERIALS. Pretreatment of a raw material ma be necessary in order to accomplish saccharification, removal of exces ions or toxic products, and adjustment of the pH.

Methods for saccharifying cellulosic and starch-containing material are described elsewhere in this text. Some special procedures are mentioned in the discussions which follow.

NITROGEN SOURCES. The usual sources of nitrogen for food-yeas propagation are ammonia, ammonium sulfate, diammonium phosphate urea, and amino acids from hydrolyzed grains.

PHOSPHORUS SOURCES. Diammonium phosphate and superphosphat were used commercially in Germany (Saeman et al., 1945). Potassiu acid phosphate and superphosphate have been used successfully in th United States (Agarwal et al., 1947).

OTHER NUTRIENTS. Potassium chloride, magnesium sulfate, or magnesium chloride may be added to raw materials containing an insufficien supply of these ingredients.

Special sources of accessory growth substances may be added to medi in which *S. cerevisiae* is cultivated (refer to the foregoing section o bakers' yeast).

*Propagation Conditions.* The control of variables in batch processes for propagating food yeast is very similar to that exercised in the production of bakers' yeast. However, there are differences in the continuous operation, which will be referred to later.

*pH.* The initial pH is generally adjusted to approximately 4.5, depending upon the raw materials used and the conditions of fermentation. Vincenty and Lewis, Stubbs, and Noble adjusted their media to an initial pH of 4 to 5; Peterson, Snell, and Frazier (1945), to an initial pH of 4.5 to 5.5; and Agarwal, Singh, King, and Peterson (1947), to an initial pH of 4.6.

The control of pH may be accomplished with an automatic pH controller and recorder or manually.

TEMPERATURE. The temperature employed will be that which provides the highest yields with the least amount of difficulty. Ordinarily, a range of 25 to 30°C. is satisfactory; however, temperatures up to 37°C. have been used occasionally.

AERATION. This subject has been discussed in the section on bakers' yeast.

FOAMING. Excessive foaming must be controlled by the use of chemical antifoam agents or mechanical defoaming equipment, coupled with adjustment of the aeration rate.

The antifoam agent used should be nontoxic to the yeast and ultimate consumer, effective in low concentration, stable under the conditions of use, and low in price. Likewise, it should not interfere with the recovery of the product.

Among antifoam agents which have been used are Vegifat Y (Peterson et al., 1945), DC Antifoam A (Kauppi, 1951), dodecanol (Steibelt, 1937), ulfonated castor oil (Walker and Morgen, 1946), and equal parts of hexadecanol or tetradecanol and paraffin oil (Keipper, 1950).

Mechanical defoamers are excellent. They were first used in the Waldhof process (Saeman et al., 1945) and are used in continuous procsses in general.

*Yields.* The yields obtained depend upon the yeast used, the medium, and the conditions of propagation. Yields (dry yeast) of 53 to 64 per cent based on the sugar present are not uncommon. Table 21 summarizes some data concerning yields and the protein content of yeasts produced by different investigators.

**Production from Various Raw Materials.** Examples of methods used to produce food yeast follow.

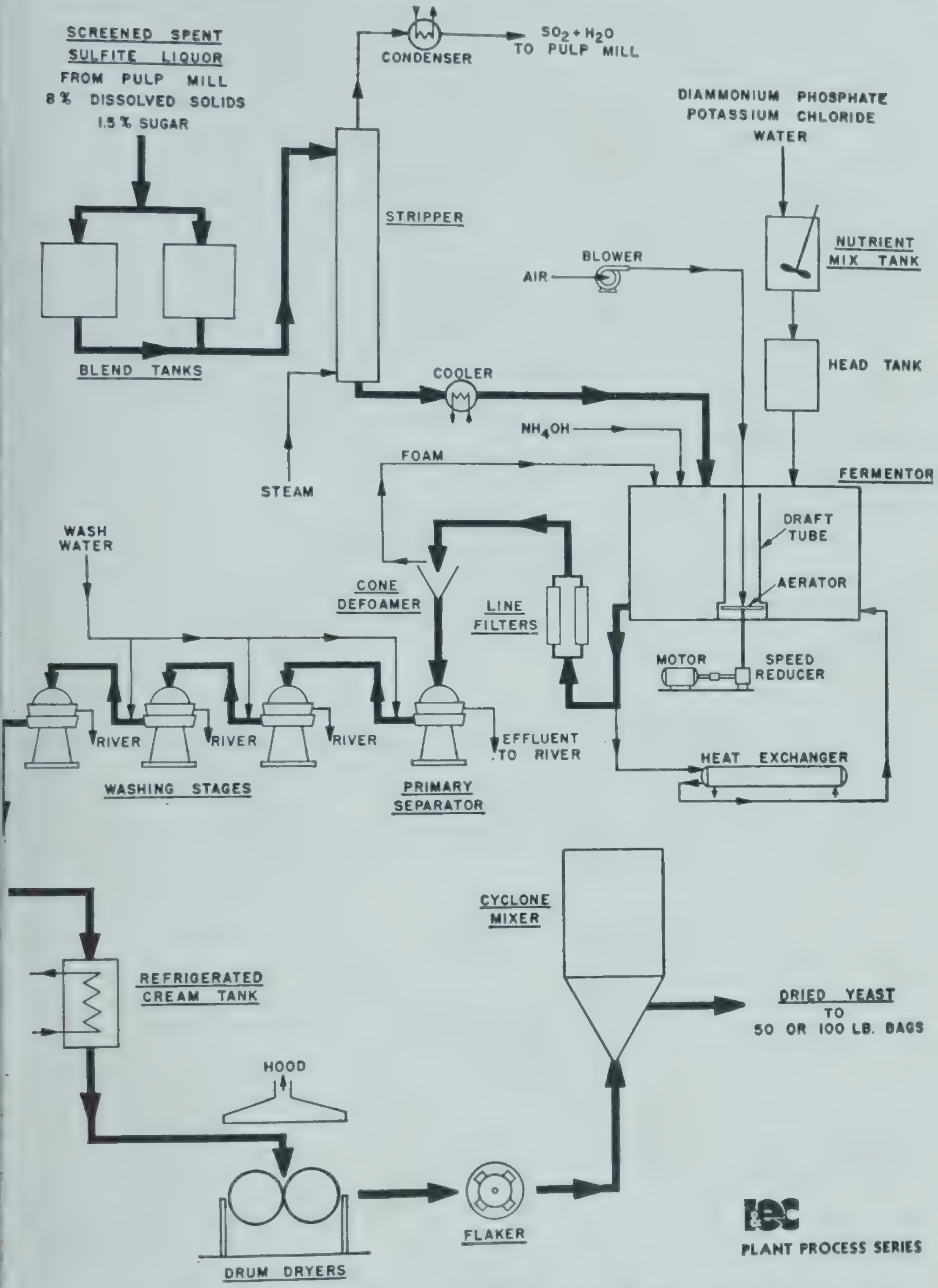
*Commercial Manufacture from Sulfite Liquor.* The description which follows is based on the report by Inskeep, Wiley, Holderby, and Hughes (1951). A flow sheet for this process is shown in Fig. 12.

The raw material is sulfite liquor from a pulp mill. This contains pproximately 8 per cent dissolved solids and 1.5 per cent sugars (about 0 per cent hexoses and 20 per cent pentoses) at a pH of about 2.5 and t a temperature of about 76°C. It is filtered through an 80-mesh tainless-steel screen mounted on a wooden frame and stored in wooden lend tanks of 125,000-gal. capacity. The hot liquor is pumped from the

TABLE 21. YIELDS AND PROTEIN CONTENTS OF YEASTS GROWN IN DIFFERENT MEDIA

| Yeast | Medium | Dry-yeast yield, per cent | Protein content, per cent | Reference |
|---|---|---|---|---|
| *Candida utilis* No. 3 | Wood hydrolyzates | 35–40 * | ............ | Peterson, Snell and Frazier: *Ind. Eng. Chem.*, **37**: 30 (1945 |
| *C. utilis* No. 3 | Wood hydrolyzates | 30.5–52.6 † | 51.9–58.6 | Harris, Saeman, Marquardt, Hannan, an Rogers: *Ind. Eng. Chem.*, **40**: 1220 (1948). |
| *C. utilis* No. 3 | Sulfite liquor | 29.6–39.2 † | 51.0–52.7 | Harris, Saeman, Marquardt, Hannan, an Rogers: *Ind. Eng. Chem.*, **40**: 1220 (1948). |
| *C. utilis* No. 3 | Fermentation residue | 47.0–52.0 † | 50.0 | Harris, Saeman, Ma quardt, Hannan, an Rogers: *Ind. Eng. Chem.*, **40**: 1220 (1948). |
| *C. utilis* No. 3 | Wood sugar stillage | 53–63 ‡ | 52.9 | Kurth and Cheldelin *Ind. Eng. Chem.*, 3 617 (1946). |
| *C. utilis* (Anheuser-Busch) | Molasses (cane) Puerto Rican | 45.1–48.4 § | 33.1–50.8 | Vincenty: *Mass. Ins Technol. Research Rept.*, Feb. 18, 194 |
| *C. utilis* | Molasses (beet) | 53.5–65.3 † | 43.7–60.6 | Agarwal, Singh, Kin and Peterson: *Arc Biochem.*, **14**(1 and 2): 105 (1947). |
| *C. utilis* | Molasses | 56 † | Av. over 50 ¶ | Lewis, Stubbs and Noble: *Arch. Biochem.*, **4**(3): 3 (1944). |
| *C. utilis* | Fruit juice | 53 † | Av. over 50 ¶ | Lewis, Stubbs and Noble: *Arch. Biochem.*, **4**(3): 3 (1944). |
| *C. utilis* var. major | | 59–62 ‡ | 56 | Thaysen and Morris *Nature*, **152**(3862): 526 (1943). |
| *C. arborea* | Molasses (beet and cane) | 55–64 † | 38.8–49.4 | Agarwal, Singh, Kin and Peterson: *Arc Biochem.*, **14**(1 an 2): 105 (1947). |
| *Hansenula anomala* | Wood hydrolyzates | 35–40 * | ............ | Peterson, Snell and Frazier: *Ind. Eng. Chem.*, **37**: 30 (194 |
| *H. suaveolens* Y-838 | Wood sugar stillage | 53–63 ‡ | 53.4 | Kurth and Cheldeli *Ind. Eng. Chem.*, 3 617 (1946). |
| *Mycotorula lipolytica* P-13 | Wood hydrolyzates | 35–40 * | ............ | Peterson, Snell and Frazier: *Ind. Eng. Chem.*, **37**: 30 (194 |
| *M. lipolytica* P-13 | Wood sugar stillage | 53–63 ‡ | 51.0 | Kurth and Cheldeli *Ind. Eng. Chem.*, 3 617 (1946). |
| *Geotrichum candidum* | Molasses (beet and cane) | 55.8–60.0 † | 31.2–41.9 | Agarwal, Singh, Ki and Peterson: *Ar Biochem.*, **14**(1 an 2): 105 (1947). |
| *Saccharomyces cerevisiae* | Molasses (beet and cane) | 42.7–54.3 † | 42.5–53.1 | Agarwal, Singh, Ki and Peterson: *Ar Biochem.*, **14**(1 an 2): 105 (1947). |

* Based on total reducing sugars.
† Based on total sugar.
‡ Based on sugar consumed.
§ Based on sugar in molasses.
¶ In most cases.


SCREENED SPENT SULFITE LIQUOR
FROM PULP MILL
8% DISSOLVED SOLIDS
1.5% SUGAR
BLEND TANKS
STRIPPER
STEAM
CONDENSER
$SO_2 + H_2O$ TO PULP MILL
DIAMMONIUM PHOSPHATE
POTASSIUM CHLORIDE
WATER
NUTRIENT MIX TANK
HEAD TANK
BLOWER
AIR
COOLER
$NH_4OH$
FOAM
FERMENTOR
DRAFT TUBE
AERATOR
MOTOR
SPEED REDUCER
HEAT EXCHANGER
LINE FILTERS
CONE DEFOAMER
WASH WATER
RIVER
RIVER
RIVER
EFFLUENT TO RIVER
WASHING STAGES
PRIMARY SEPARATOR
REFRIGERATED CREAM TANK
HOOD
DRUM DRYERS
FLAKER
CYCLONE MIXER
DRIED YEAST TO 50 OR 100 LB. BAGS
I&EC
PLANT PROCESS SERIES

. 12. Flow sheet for production of food yeast from sulfite liquors at the Rhine-
der, Wis., plant of Lake States Yeast Corp. [*Courtesy of G. C. Inskeep, A. J.*
*ley, J. M. Holderby, and L. P. Hughes, Ind. Eng. Chem.*, 43: 1702 (1951). *Copy-*
*ht* 1951 *by the American Chemical Society and reprinted by permission of the*
*yright owner.*]

blend tank to the top of a steam stripper, which reduces its sulfur dioxid content.

The stripper is 5 ft. in diameter and 22 ft. tall. It is packed wit Karbate 1- by 1-in. Raschig rings. Steam is admitted to the bottom the stripper through a sparge drill. Sulfur dioxide and water vapors ar condensed in a condenser and returned to the paper mill to be used agai The stripped sulfite liquor passes out through the bottom of the sti through a control valve (automatic-level type) at about 80°C. It cooled to about 26.7°C. by passage through a stainless-steel cooler an admitted to a fermentor at a predetermined rate.

The fermentor, constructed of carbon steel, is clad with stainless stee open at the top, 26 ft. in diameter and 14 ft. high, and equipped with central draft tube 4 ft. in diameter. It has a capacity for 20,000 gal. o mash or 45,000 gal. of emulsion.

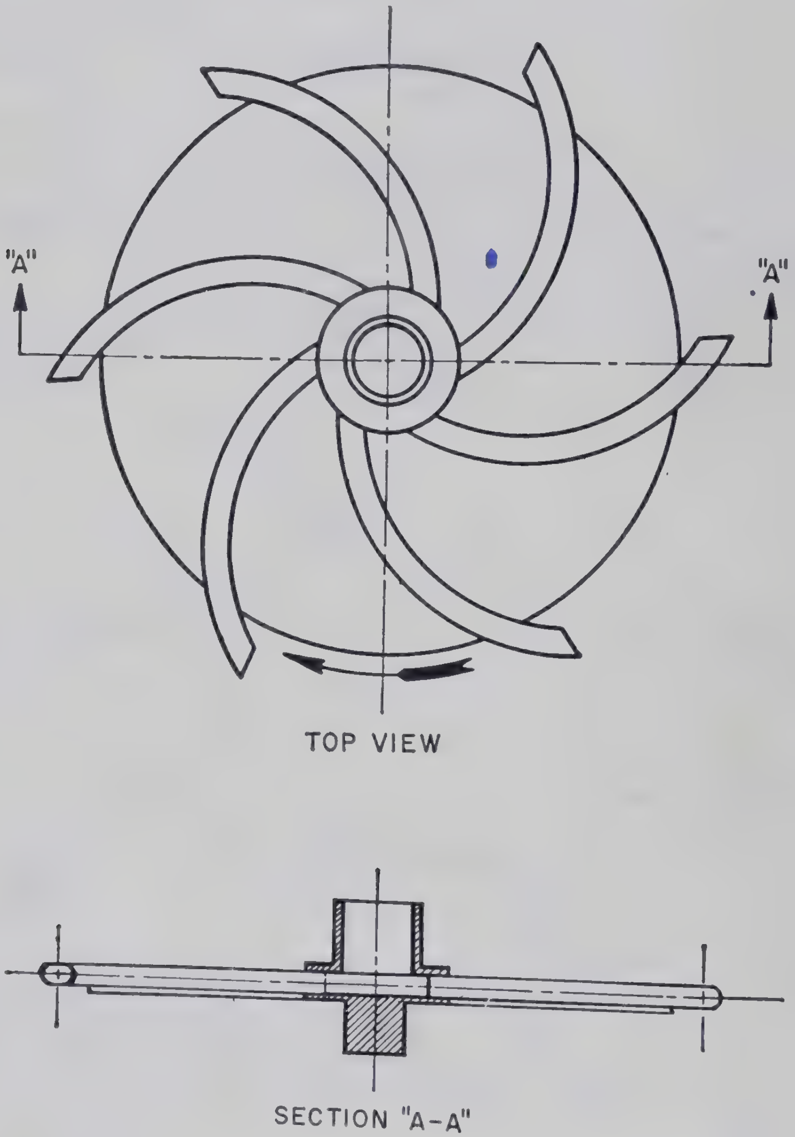


FIG. 13. Principle of the Waldhof aeration wheel. Modification of this type o aerator is used in the yeast fermentor at Lake States Yeast Corp. [*Courtesy of G. C Inskeep, A. J. Wiley, J. M. Holderby, and L. P. Hughes, Ind. Eng. Chem.*, **43**: 170 (1951). *Copyright* 1951 *by the American Chemical Society and reprinted by permission of the copyright owner.*]

Air is supplied at the bottom of the draft tube through a modified aldhof aeration wheel (refer to Fig. 13), which possesses open-end bes. The wheel is rotated at 300 r.p.m. Air (unsterilized) is supplied y a centrifugal blower (at a rate of up to 1,700 c.f.m.) and conducted the aeration wheel through an 8-in. pipe located in the center of the raft tube. The wheel, in combination with the draft tube, acts as a echanical defoaming agency. The wort containing the yeast cells in spension moves downward in the interior of the draft tube and upward round its exterior.

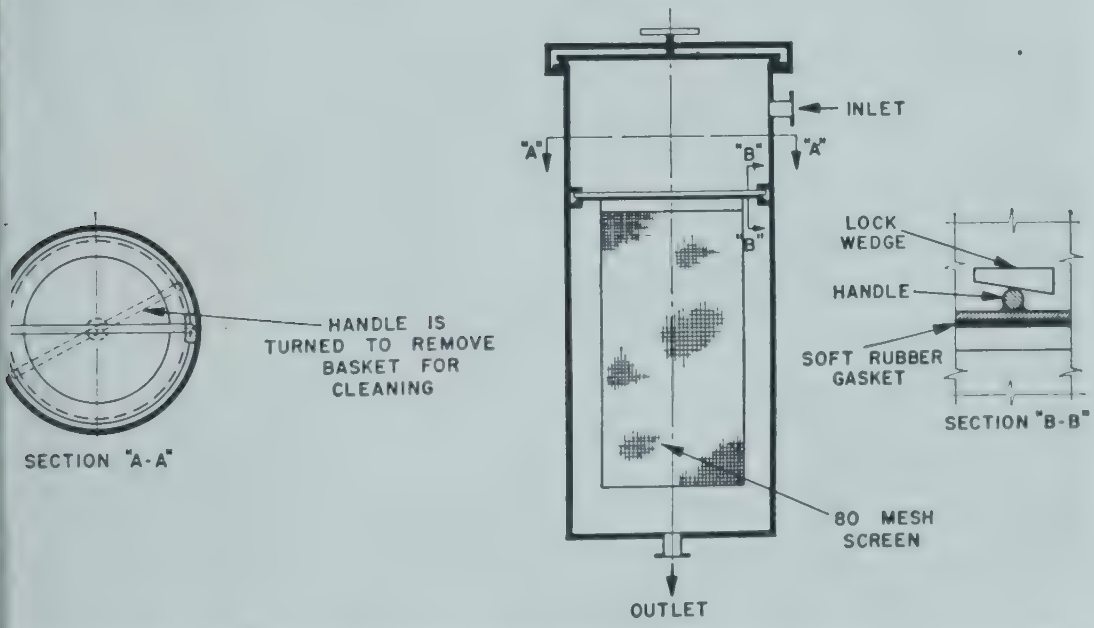
The nutrients, diammonium phosphate and potassium chloride, are ixed with water in a 500-gal. tank and pumped to a head (surge) tank. hey are fed continuously by gravity to the fermentor from the head tank.

An aqueous solution of ammonia, containing about 20 per cent of nmonia, is also fed continuously into the fermentor at a rate which is rrelated with the sugar content of the sulfite liquor. The pH of the ort is about 5.

Fermentation is carried out at 37°C., and a shell-and-tube heat changer is used to control the temperature. The wort is removed om one side of the bottom of the fermentor, passed through the heat changer, and returned to the other side of the fermentor.

The process is a continuous one, in which contamination has not been problem.

Since this is a continuous process, some of the yeast suspension is moved continuously. Yeast suspension on its way to the heat exchanger diverted from the main stream at a rate (volume) equal to that of the tal feed of the fermentor. It is passed through a two-screen type of ter (refer to Fig. 14), one screen of which may be removed for cleaning



. 14. Cross section of line filters used to clarify feed to yeast separators. The r was designed and built by Lake States Yeast Corp. [*Courtesy of G. C. Inskeep, I. Wiley, J. M. Holderby, and L. P. Hughes. Ind. Eng. Chem.*, 43: 1702 (1951). *yright* 1951 *by the American Chemical Society and reprinted by permission of copyright owner.*]

purposes without interrupting the filtration process, to a cone defoamer. Foam from the top of the latter is returned to the fermentor. The yeast suspension, taken from the bottom of the cone, flows by gravity to a centrifugal separator. The effluent from the separator, which is relatively free of sugar, is discharged into a river. The yeast cream is washed with well water in three stages. The washed yeast is collected in a cream storage tank (which is refrigerated when it becomes necessary for any reason to keep the yeast for a while) and agitated until delivered to twin rotary driers. After drying, the yeast is collected in a trough equipped with a screw conveyor, transported by a bucket conveyor to a flaker, and carried by means of a screw conveyor after flaking to a cyclone mixer. The product, which does not contain viable cells, possesses a moisture content of about 6 per cent. It is packaged in multiwall paper bags in 50- and 100-lb. amounts.

Food yeast was produced commercially from sulfite liquors in Germany during World War II, principally by the Waldhof process (Saeman et al., 1945). The standard plant using this process had a capacity of 1,000 tons of yeast (7 per cent moisture) per year. The process was rapid and continuous and employed mechanical aeration and foam-breaking equipment. The cooling coils used to maintain a uniformly desirable temperature are shown in Fig. 15.

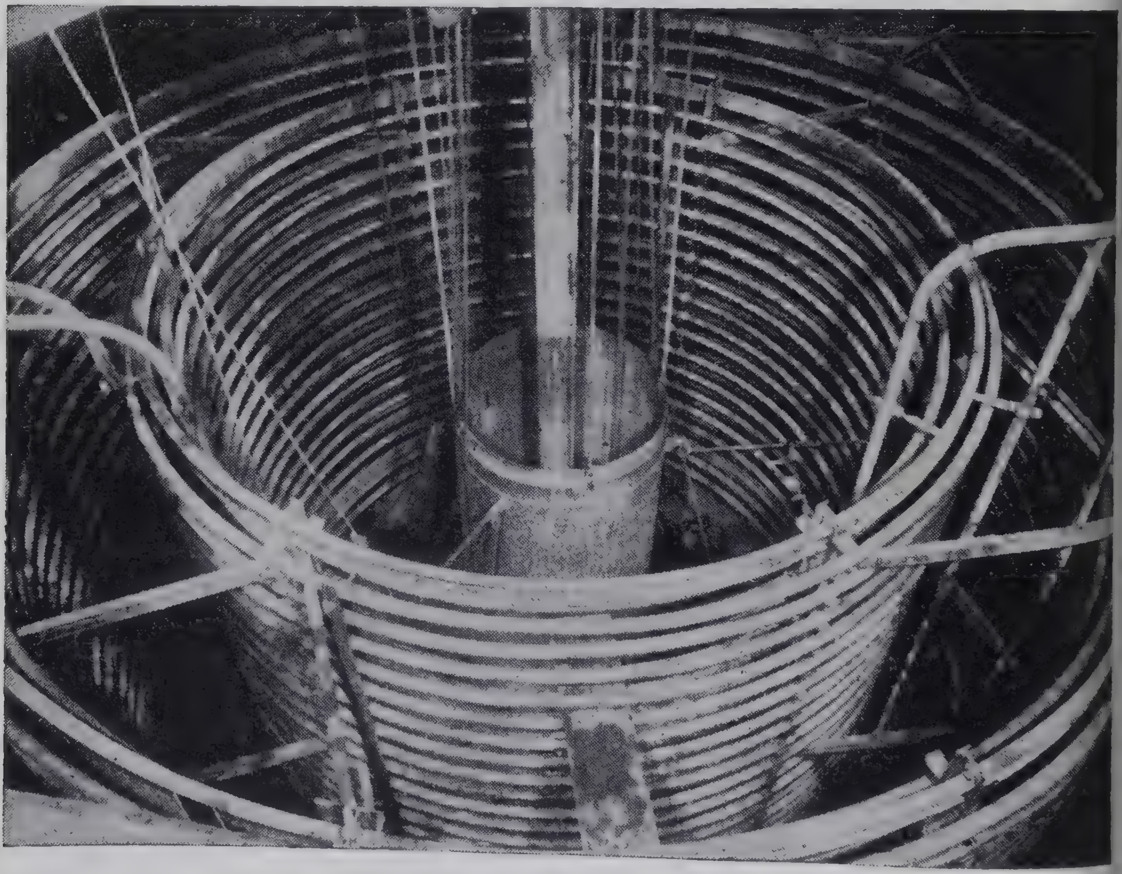


FIG. 15. Cooling coils in fermentor for yeast propagation. [*Courtesy of J. F. Saeman, E. G. Locke, and G. K. Dickerman, FIAT Final Report No.* 499, *Nov.* 14, 1945; *also E. G. Locke, Pulp & Paper,* 20(1): 20 (1946).]

*Laboratory Production from Sulfite Liquor.* Laboratory-scale studies on food- or fodder-yeast production from waste sulfite liquors have been carried out by a number of scientists. Walker and Morgen (1946), Harris and associates (1947), and others have contributed basic knowledge concerning production.

*Production from Wood Sugar.* According to Saeman, Locke, and Dickerman (1946), 8,985 tons of yeast were produced in Germany in 1944 by five plants which used sugar obtained by the acid hydrolysis of wood. The wood sugar was produced by the Bergius and Scholler methods.

The process, as carried out at the Regensburg Vogelbusch plant, was as follows: Propagation was conducted in six 250-cu. m. Vogelbusch fermentors, each of which was equipped with internal cooling coils and a 20 KVA motor which drove the propeller at 8 r.p.m.

The organism used was *C. utilis.* The inoculum for a fermentor was obtained from a previous fermentation. On the basis of the sugar being used, a quantity equivalent to 10 to 20 per cent by weight of dry yeast was employed.

Propagation was carried out at 35°C. For each kilogram of yeast produced, 20 cu. m. of air was supplied.

The suspension of yeasts was centrifuged in a battery of Westphalia centrifuges of the direct-drive type. The cream thus obtained was washed by dilution with water, centrifuged again, concentrated to a 0 per cent cake with a rotary filter, and dried on Oschatz double-drum riers.

The yield of yeast was 45 to 48 per cent based on the sugar.

For the manufacture of 500 tons of yeast per month at the Regensburg lant, the following chemical materials were necessary, according to aeman, Locke, and Dickerman:

| Material | Tons |
|---|---|
| Ammonium sulfate | 10 |
| Liquid ammonia | 75 |
| Potassium chloride | 15 |
| Diammonium phosphate | 30 |
| Magnesium sulfate | 8 |
| Trisodium phosphate | 0.5 |
| "Garfett" | 7.5 |
| Lipon (another antifoam) | 9.5 |

Peterson, Snell and Frazier (1945) studied approximately 150 fermentations of hydrolyzates from 13 different species of wood in which nine ypes of yeasts were used.

These investigators prepared the wood hydrolyzates for fermentation four different procedures, but the following one was most successful. he amounts of the various materials used were based on 5 to 6 per cent ducing sugar. Calcium carbonate was added to the hydrolyzate until e pH was 5 (approximately 0.75 g. per 5 g. reducing sugar). Next, 05 per cent of sodium sulfite was added. The liquor was then heated to iling with live steam, cooled, and filtered by passage through a pad of -Flo Supercel. The nutrient requirements were satisfied by adding

0.06 g. of urea and 0.05 g. of potassium dihydrogen phosphate for each gram of reducing sugar present. The medium was then diluted to the desired concentration.

The standard laboratory procedure for yeast propagation by Peterson and his associates was as follows: A 6,300-ml. amount of the medium was placed in a 5-gal. Pyrex bottle. It was inoculated with 700 ml. of an inoculum which contained 1 g. of cells per 100 ml. A canvas aerator was placed "well below the surface of the medium." To prevent excessive foaming, 10 ml. of Vegifat Y was added. The Pyrex bottle was introduced into a constant-temperature bath (or incubator) at 30°C. Saturated air at the rate of 20 to 40 liters per min. was passed through the aerator into the medium. The propagation was allowed to continue for 24 hr.

Yields of 35 to 40 per cent of dry yeast, based on the total reducing sugars, were obtained from such yeasts as *C. utilis* No. 3, *Mycotorula lipolytica* P-13, and *Hansenula anomala.* Approximately 90 per cent of the apparent reducing sugar was utilized.

*Production from Molasses, Sugar Juice, and Raw Cane Sugar.* Commercial production of food yeast from molasses, sugar juice, and raw cane sugar has been carried out in a plant located at Frome, Jamaica, West Indies. This project was undertaken by the Colonial Office of Great Britain as a result of the need for such a product and of the information and experience obtained at the pilot plant operated at Teddington, England, by the Department of Scientific and Industrial Research. A description of some of the progress made at Teddington and at Jamaica has been furnished by the Colonial Food Yeast, Ltd. (1944), by Thaysen (1945), and by others.

*Production from Wood Sugar Stillage.* Kurth (1946), Kurth and Cheldelin (1946), Harris and associates, and others have studied the production of fodder yeasts from wood sugar stillage on a laboratory basis. Wood sugar stillage (still waste liquor) is the product remaining after wood sugar hydrolyzates are fermented with yeasts and subsequently distilled and contain pentoses (xylose and arabinose), acids (acetic, formic, etc.), ash, and other constituents. The compositions of the waste liquor obtained from a Douglas fir hydrolyzate and the spent liquors after fermentation are shown in Table 22.

The yeasts used by Kurth and Cheldelin were *C. utilis* No. 3, *M. lipolytica* P-13, and *H. suaveolens* Y-838. They were transferred repeatedly in a wood stillage medium to increase their rate of growth. As inocula, yeast concentrations of about 100 million cells per ml. were desirable.

The conditions of fermentation were as follows: The medium contained wood sugar stillage from Douglas fir, reinforced by the addition of urea and diammonium hydrogen phosphate in 0.05 per cent concentrations. The pH was maintained between 5 and 6.5 with ammonia. After inoculation the liquor was aerated. The rate of growth of the yeast and also of the sugar consumption was affected to the largest extent by aeration. It was discovered that it was most important to use small-sized air bubbles for best results. Table 23 shows the effect of aeration on propagation time and final reducing-sugar content. With suitable aeration the

TABLE 22. ANALYSIS OF LIQUOR BEFORE AND AFTER *Torula* GROWTH *

| | Still waste liquor | *Torula* spent liquors |
|---|---|---|
| Total solids, per cent | 3.2 | 2.40 |
| Reducing sugar (xylose), per cent | 0.81 | 0.20 |
| Reducing sugar after hydrolysis with $H_2SO_4$, per cent | 0.97 | 0.34 |
| Volatile acid (acetic), per cent | 0.24 | 0.10 |
| Volatile and nonvolatile acid (acetic), per cent | 0.78 | 0.31–0.5 |
| Ash, per cent: | | |
| Residue upon combustion | 0.65 | 0.36–0.47 |
| Residue converted to sulfate | 1.03 | 0.58–0.68 |
| Total carbon, per cent | 1.42 | 0.94–1.04 |
| -day B.O.D. | 16,400 | 9,600 |
| H | 5.0 | 7.5–8.0 |

* E. F. Kurth, *Ind. Eng. Chem.*, 38(2): 204 (1946). Copyright 1946 by the Ameri-an Chemical Society and reprinted by permission of the copyright owner.

TABLE 23. EFFECT OF AERATION *

| Aerator | Time, hr. | Final reducing sugar, per cent |
|---|---|---|
| -porosity fritted-glass tubes | 16 | 0.30 |
| | 19 | 0.20 |
| oth or EC-porosity fritted-glass disks | 24 | 0.20–0.22 |
| mm. glass tubing | 24 | 0.40–0.49 |
| aker flasks | 72 | 0.30 |

* E. F. Kurth, *Ind. Eng. Chem.*, 38: 204 (1946). Copyright 1946 by the American emical Society and reprinted by permission of the copyright owner.

similable sugar may be consumed in 18 hr. or less. The rate of sugar nsumption was increased as the temperature was increased from 24 34°C.

Kurth and Cheldelin found that there was but little distinction among e different species of yeasts used in respect to their rate of growth, gar utilization, yield, and nutritional value. All were able to utilize a rge proportion of the residual sugars and acids in the wood sugar llage. Yields of dry yeasts varied from 53 to 63 per cent on the basis the sugar consumed.

Data concerning the contents of protein, amino acids, and B vitamins the three yeasts studied by Kurth and Cheldelin are given in Tables 24 d 25.

*Fodder Yeast from Citrus Waste Press Juice.* Nolte, von Loesecke, d Pulley (1942) described the production of fodder yeast from citrus

TABLE 24. AMINO ACIDS AND PROTEIN CONTENT OF YEASTS

(Percentage of oven-dry weights)*

| Yeast | *Mycotorula* | *Hansenula* | *Torula* | Brewers'† |
|---|---|---|---|---|
| Total protein | 51.0 | 53.4 | 52.9 | 51.8 |
| Amino acid:‡ | | | | |
| Arginine | 3.2 | 2.9 | 3.1 | 2.7 |
| Histidine | 1.4 | 1.4 | 1.5 | 1.3 |
| Lysine | 4.4 | 4.3 | 4.4 | 3.5 |
| Phenylalanine | 2.4 | 2.4 | 2.3 | 2.4 |
| Tryptophan | 0.3 | 0.3 | 0.3 | 0.8 |
| Threonine | 2.5 | 2.4 | 2.5 | 2.8 |
| Leucine | 3.7 | 3.6 | 3.8 | 3.7 |
| Isoleucine | 3.5 | 3.7 | 3.7 | 3.1 |
| Valine | 3.1 | 3.3 | 3.3 | 2.4 |

* E. F. Kurth and V. H. Cheldelin, *Ind. Eng. Chem.*, **38**: 617 (1946). Copyright 1946 by the American Chemical Society and reprinted by permission of the copyright owner.

† Reported by R. J. Block and D. Bolling, *Arch. Biochem.*, **7**:313 (1945), for strain K 105.

‡ Values are expressed in terms of the naturally occurring isomer.

TABLE 25. B-VITAMIN CONTENT OF YEASTS

| | Vitamin content, $\gamma$/g. dry wt.* | | |
|---|---|---|---|
| | *Mycotorula* | *Hansenula* | *Torula* |
| Thiamin | 5.3 | 8.5 | 6.2 |
| Riboflavin | 59 | 54 | 49 |
| Nicotinic acid | 600 | 590 | 500 |
| Pantothenic acid | ..... | 180 | 130 |
| Biotin | 1.8 | 1.7 | 1.8 |
| Folic acid | 3.1 | 1.7 | 2.8 |
| *p*-Aminobenzoic acid | 31 | 16 | 17 |

* E. F. Kurth and V. H. Cheldelin, *Ind. Eng. Chem.*, **38**: 617 (1946). Copyright 1946 by the American Chemical Society and reprinted by permission of the copyright owner.

waste press juice. This juice is obtained by pressing ground citrus waste (peel, rag, and seeds) that has been limed. It contains 4.08 to 8.58 per cent of total sugars with an average of 6.63 per cent. It is deficient in phosphates and nitrogen.

The juice is prepared for use by screening it, heating it to the boiling temperature, and holding it for 5 min., adding filter aid to it and filtering, diluting it with tap water to adjust the total sugar content to 1 per cent, and cooling to 30°C. Trisodium phosphate and ammonium sulfate are added to the juice in the proportion of 9 per cent based on the total amount of sugar in the batch. Since additional ammonium sulfate is required, it is added during the process at the end of 1 hr. and during the ucceeding 3 hr. at the rate of 1.75 per cent per hr. Sufficient sodium arbonate is added to maintain the pH between 4.4 and 6.5.

The nutrient citrus waste press juice is inoculated with 4 per cent by olume of a 2-day culture of *C. utilis* in malt wort (which contained per cent of total carbohydrates at the time of inoculation). The medium s aerated vigorously with filtered air and maintained at a temperature f 29 to 30°C. The process is generally completed in 8 hr. The yeast s separated by centrifuging and washed. The yields from citrus waste uice containing 1 per cent of total sugar (after dilution) are 44.3 to 48 er cent of dry yeast (177 to 191.9 per cent of wet yeast containing 5 per cent of solids) based on the total amount of sugar in the juices.

*Production from Waste Protein Water.* Based on the results of labora-ory experiments, it has been shown that food yeast of 55 per cent protein ontent may be produced from the protein waste water of potato starch lants, with a 60 per cent or greater reduction in the 5-day B.O.D. when 0 per cent of the solids was recovered (Reiser, 1954). The yeast, *C. utilis* RRL Y-900, was capable of utilizing as much as 40 per cent of the olids of the protein waste water without the addition of nutrients. Con-itions favoring good yields of yeast included the use of a pH of 5, a mperature of 30 to 32°C., aeration, agitation, and Swift's inedible efoamer No. 1000.

*Production from Olive Residue Hydrolyzate.* Food yeast may be pro-uced effectively (on a laboratory basis) from the neutralized hydrolyzate f olive residue from the olive-oil plant (Tsilensis and Hedrick, 1952). he olive residue was pulverized and treated twice with 3.5 per cent lfuric acid (liquid/solids ratio of 5:1) at a temperature of 120°C. for hr. The hydrolyzate thus obtained was neutralized with anhydrous lcium carbonate, using intermittent boiling and filtration. Yeast extract malt extract (or both) was used to supply the nutrients. Yeasts grew the nutrient hydrolyzate in the following approximate descending order effectiveness: *C. utilis, Pichia membranaefaciens, Geotrichum, S. carls-rgensis,* and *C. krusei.* Yields of 50 to 65 per cent of dry yeast, based the sugar utilized, were obtained. It was estimated that 4.5 to 5.5 per t of the olive residue may be converted to protein by this method.

*Production of Fodder Yeast on a Continuous Basis.* The continuous opagation of yeast has been studied by Harris and his fellow re-archers. The equipment used was a modification of the Waldhof fer-ntor, which was used in Germany during World War II for the ntinuous propagation of food yeast from sulfite liquors. It consisted an open tank of 34-liter fermentation-medium capacity; a mechan-lly driven spinner, 6 in. in diameter, which was operated at a speed 1,100 r.p.m. and served as a combination aerator and agitator by wing the fermentation liquor down through a draft tube and forcing it and around the sides; a proportioning pump to control continuous ding; and a standpipe, which was slightly higher than the draft tube, controlling continuous discharge.

The propagation was carried out as follows: 2 liters of a solution that contained 100 million yeast cells per ml. (*C. utilis* No. 3) were placed in the fermentor. The spinner was put into operation and then wood sugar hydrolyzate, diluted to a concentration of 2.5 per cent of reducing sugars and containing nutrients, was added at a rate of 2 liters per hr. Air was introduced at the rate of 0.5 c.f.m. The spent medium containing the yeast started to overflow from the fermentor into the standpipe in about 5 hr. The yeast was separated from the liquor by centrifuging and returned to the fermentor until the volume of wet yeast in the fermentor was about 7 per cent, or 1.8 per cent dry yeast. At this point, undiluted wood hydrolyzate, of 4.5 to 5 per cent concentration, was admitted, and the rate of air flow was increased to 0.75 c.f.m. The temperature of fermenta-

TABLE 26. CONTINUOUS PROPAGATION OF *Candida utilis* ON WOOD HYDROLYZATE, ON SUGAR RESIDUES FROM ALCOHOLIC FERMENTATION OF WOOD SUGARS, AND ON SULFITE LIQUOR *

| Series no. | Rate of feed, liters/hr. | Sugar concentration, per cent | pH | Air, c.f.m. | Air, cu. ft./lb. of yeast | Reducing sugar used, per cent | Dry-yeast yield based on total sugar, per cent | Protein content of yeast, per cent |
|---|---|---|---|---|---|---|---|---|
| Wood hydrolyzate | | | | | | | | |
| 9 | 3.0 | 4.5 | 4.7 | 0.75 | 415 | 88.4 | 36.4 | 52.0 |
| 10 | 2.0 | 4.5 | 5.0 | 0.75 | 630 | 92.0 | 35.9 | 52.3 |
| 11 | 4.0 | 4.5 | 5.0 | 0.75 | 313 | 86.5 | 36.0 | 52.7 |
| 12 | 4.5 | 4.5 | 5.0 | 0.75 | 275 | 85.3 | 36.5 | 54.4 |
| 13 | 5.0 | 4.5 | 5.0 | 0.75 | 296 | 83.1 | 30.5 | 52.5 |
| 14 | 3.0 | 4.6 | 6.6 | 0.75 | 295 | 93.8 | 49.7 | 58.6 |
| 15 | 4.0 | 4.6 | 6.0 | 0.75 | 210 | 93.4 | 52.6 | 57.3 |
| 16 | 4.5 | 4.6 | 6.0 | 0.75 | 212 | 93.2 | 46.5 | 56.5 |
| 17 | 5.0 | 4.6 | 6.0 | 0.75 | 203 | 91.1 | 43.4 | 52.5 |
| 18 | 5.0 | 4.6 | 5.5 | 0.75 | 208 | 90.4 | 42.5 | 51.9 |
| Fermentation residues | | | | | | | | |
| 19 | 3.0 | 0.94 | 5.4 | 0.60 | 1100 | 60.0 | 52.0 † | 50.0 |
| 20 | 4.0 | 0.91 | 5.5 | 0.60 | 950 | 55.0 | 47.0 † | 50.0 |
| Sulfite liquor | | | | | | | | |
| 21 | 2.0 | 1.66 | 5.0 | 0.50 | 1040 | 79.0 | 39.2 | 52.7 |
| 22 | 3.0 | 1.66 | 5.0 | 0.50 | 695 | 78.0 | 39.0 | 52.5 |
| 23 | 4.0 | 1.66 | 5.0 | 0.50 | 540 | 75.0 | 37.6 | 52.0 |
| 24 | 5.0 | 1.66 | 5.0 | 0.50 | 550 | 59.0 | 29.6 | 51.0 |

* E. E. Harris, J. F. Saeman, R. R. Marquardt, M. L. Hannan, and S. C. Rogers. *Ind. Eng. Chem.*, 40(7): 1220 (1948). Copyright 1948 by the American Chemical Society and reprinted by permission of the copyright owner.

† Values corrected to product with 50 per cent protein.

ion was 30 to 32°C. and was controlled by cooling coils and an electric eater. No antifoam agent was required because of the type of aerator sed. The initial pH of the fermentation medium was 4.7 to 6.6; that of he feed, 4.2 to 4.5.

Results of the propagation of *C. utilis* on various types of media and nder various conditions are shown in Table 26.

**Nutritive Value.** The nutritive value of food yeast has been studied y a large number of researchers, particularly in Germany, Great Britain, nd this country. These studies have been concerned with such subjects s the digestibility of the yeast in human nutrition, its vitamin content hen grown on different media, its protein content, its amino acid content, nd its value as a food constituent for children and adults and as a feed upplement for hogs, cattle, chickens, rats, and other animals.

Among the reports on these subjects may be mentioned those of ingerling and Honcamp (1933); Bunger and associates (1934); Fink nd Just (1938, 1939); Scheunert and Wagner (1939); Stephenson, Pen- on, and Korenchevsky (1941); Dirr (1942); Dirr and Von Soden (1942); ock (1942); Bickel (1942); Thaysen (1943); Thaysen and Morris 1943); Lewis (1944); Lewis, Stubbs, and Noble (1944); Butschek 1944); Wilson (1944); Colonial Food Yeast, Ltd. (1944); Klose and evold (1945); Peters (1945); Kurth and Cheldelin (1946); and Agarwal, ingh, King, and Peterson (1947). The subject has been reviewed by arter and Phillips (1944), Dunn (1952), and others.

The high protein content of dried yeast has already been referred to Table 21). Yeast protein appears to be somewhat deficient in respect to e amino acids methionine and cystine (Hock and Fink, 1943). In ite of this fact, food yeast may be regarded as a valuable source of otein.

Information concerning the composition of food yeast may be found Chap. 2. Table 27 shows the vitamin content of three yeasts and *candidum.*

TABLE 27. VITAMIN CONTENT OF SOME YEASTS AND *Geotrichum candidum* *

(Gamma per gram of dry microbe)

| Molasses | *Saccharomyces cerevisiae* | | | | *Candida utilis* | | | | *C. arborea* | | | | *Geotrichum candidum* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Thiamin | Riboflavin | Niacin | Folic acid | Thiamin | Riboflavin | Niacin | Folic acid | Thiamin | Riboflavin | Niacin | Folic acid | Thiamin | Riboflavin | Niacin | Folic acid |
| nsing (beet) ... | 37.6 | 43.8 | 414.3 | 21.6 | 37.5 | 54.2 | 520.6 | 15.2 | 32.7 | 69.5 | 503.1 | 14.8 | 20.1 | 55.0 | 192.8 | 7.7 |
| son City<br>eet) .......... | 35.7 | 50.4 | 443.3 | 21.4 | 36.7 | 62.0 | 600.0 | 10.6 | 33.1 | 52.3 | 492.3 | 16.0 | 28.9 | 39.9 | 212.6 | 5.6 |
| d (beet) ....... | 32.7 | 45.4 | 442.8 | 19.6 | 38.1 | 54.8 | 511.3 | 11.7 | 31.3 | 53.0 | 512.3 | 17.6 | 27.2 | 42.6 | 247.5 | 7.6 |
| vanan (cane<br>ackstrap) ..... | 40.8 | 49.1 | 563.1 | 19.1 | 35.4 | 58.6 | 531.4 | 10.7 | 33.1 | 60.0 | 580.2 | 15.0 | 29.0 | 43.0 | 242.4 | 7.8 |

he media used for the growth of the yeasts were comprised of treated molasses (1 per cent sugar), per cent $KH_2PO_4$, 0.1 per cent urea, and 2 ml. corn-steep liquor. The fermentation was run for r. at 30°C.

P. N. Agarwal, K. Singh, P. S. King, and W. H. Peterson, *Arch. Biochem.*, 14(1 and 2): 105 7).

A number of studies have been made on the vitamin content of *C. utili* Lewis and his associates (1944) found 18 micrograms of thiamin, 36 μ of riboflavin, and 610 μg. of niacin per gram of yeast (dry basis) pro duced in molasses media; and 22 μg. of thiamin, 44 μg. of riboflavin, an 535 μg. of niacin per gram of yeast (dry basis) produced in fruit-juic media. Kurth and Cheldelin found 6.2 μg. of thiamin, 49 μg. of riboflavi 500 μg. of niacin, and 2.8 μg. of folic acid per gram (on a dry basis) i yeast grown in wood sugar stillage. Thaysen reported 23 μg. of thiami 54 μg. of riboflavin, and 440 to 490 μg. of niacin per gram of yeast (dr basis) grown in molasses.

Table 28 presents data on commercially produced *C. utilis.*

TABLE 28. ANALYSIS OF *Torula* YEAST PRODUCED COMMERCIALLY FROM SPENT SULFITE LIQUOR *

(All data on as-received basis)

| | Chemical analyses, per cent |
|---|---|
| Moisture, atmospheric drying, 2 hr. at 105°C. | 5.85 |
| Ash | 9.07 |
| Phosphorus, as P | 1.92 |
| Calcium, as Ca | 0.85 |
| Crude protein (N × 6.25) | 47.43 |
| Crude fat: | |
| Standard method | 1.01 |
| With prehydrolysis | 4.84 |
| Crude fiber, with HCl prehydrolysis | 0.82 |
| | Vitamin assays, γ/gram |
| Thiamin | 5.3 |
| Riboflavin | 45.0 |
| Pyridoxine hydrochloride | 33.4 |
| Pantothenic acid | 37.2 |
| Biotin | 2.3 |
| Niacin | 417.3 |
| Folic acid | 21.5 |

* Courtesy of G. C. Inskeep, A. J. Wiley, J. M. Holderby, and L. P. Hughes, *In* *Eng. Chem.*, 43: 1702 (1951). Copyright 1951 by the American Chemical Society ar reprinted by permission of the copyright owner.

**Processing of Brewers' Yeast for Use as Food.** Brewers' yeast mu be debittered and otherwise treated before use as a food constituen Reports concerning the processing of brewers' yeast have been presente by Siebel, Weber, and Singruen (1941, 1942); Haffenreffer (1943 Burton (1943); MacDonough and Haffenreffer (1944); and others.

In the process described by MacDonough and Haffenreffer, yeast crea (10 per cent yeast on a dry basis) is pumped through a filter into weighing tank where it is weighed prior to transfer to a debittering tan The yeast is washed first with water, 10,000 lb. of water being added 5,000 lb. of yeast slurry. The mixture is separated into a yeast conce trate and waste liquor by passage through an Alpha Laval yeast separa

r. The yeast is washed next with a caustic soda solution at a pH of out 12.1. Two parts by weight of alkaline water are employed for each rt of yeast. The mixture is passed through the separator. The yeast is w washed with a solution containing 0.05 per cent caustic soda (5 lb. of dium hydroxide in 10,000 lb. of water) and the mixture passed through e separator. Finally the yeast is washed with cold water and concen- ated by passage through the separator.

After the debittering treatment, table salt is added to the concentrate the amount of 2 per cent of the dry weight of the yeast. Likewise fficient U.S.P. phosphoric acid is added to lower the pH to 5.5 to 5.7.

The yeast preparation may be enriched, if desired, by the addition of a lution containing thiamin, riboflavin, and niacin. The concentrate is irred uniformly and then dried on a double-drum drier. The final oduct is pulverized and packaged.

In order to prevent vitamin losses during processing it is essential to e speed and temperatures not above 40°F. These precautions are pecially important during the debittering process.

## FAT PRODUCTION BY YEASTS

Only under conditions of a national emergency would one ordinarily tempt to produce fat from microorganisms, for normally the demands of country can be met by domestic production from animals and higher ants or by importations. But when the usual supply becomes inade- ate, waste carbohydrate materials may be used for the synthesis of fat microorganisms.

**"Fat" Defined.** The term "fat," as used in this chapter, refers to bstances insoluble in water but soluble in ether and other fat solvents. the literature, one finds that some authors use the term "lipoid" to signate the crude fat obtained from microorganisms. However, in view the wide use of the word "fat," which is more properly restricted to an the esters formed by combinations of fatty acids with glycerol, this m will be used throughout the present discussion to include what actu- y would be classified as lipids, according to Bloor.[1]

Under the term "lipid," Bloor includes the simple lipids—the fats, oils, d waxes; the compound lipids, such as the phospholipids; and the rived lipids, such as fatty acids, sterols, and alcohols.

**Nature of Yeast Fat.** Yeast contains glycerides of fatty acids, phos- olipids, and sterols. The fatty acids found include myristic, palmitic, aric, hexadecanoic, oleic, linoleic, linolenic, saturated $C_{20}$ and $C_{22}$, and ssibly others in small amounts (Hoogerheide, 1948). The phospholipids rmally constitute only 1 to 2 per cent of the weight of dry yeast and made up of about 75 per cent lecithin and 25 per cent cephalin. rols include ergosterol (the most important) and small amounts of nosterol and cerevisterol.

---

H. B. Bull, "The Biochemistry of Lipids," John Wiley & Sons, Inc., New York, 7.

**Fat-rich Yeasts.** Although fat may be produced by many microorganisms when cultivated under special conditions, there are in natur certain microbes which are capable of producing large amounts of fat Some of the fat-rich microbes which have been studied extensively o which have been used industrially during World Wars I and II are liste (with their synonyms) in Table 29. Some data relating to raw material

TABLE 29. SOME MICROORGANISMS CAPABLE OF PRODUCING RELATIVELY LARGE AMOUNTS OF FAT *

| Yeasts | Molds | Bacteria |
|---|---|---|
| Genus *Candida:* | Genus *Aspergillus:* | Genus *Aerobacter* |
| *C. pulcherrima* | *A. fischeri* | *A. cloacae* |
| (*Torula pulcherrima*) | *A. nidulans* | |
| (*Torulopsis pulcherrima*) | | |
| *C. reukaufii* | Genus *Fusarium:* | |
| (*Nectaromyces reukaufii*) | *F. lini* | |
| *C. utilis* | *F. lycopersici* | |
| (*Torula utilis*) | | |
| (*Torulopsis utilis*) | Genus *Geotrichum:* | |
| | *G. candidum* | |
| Genus *Lipomyces:* | (*Oïdium lactis*) | |
| *L. lipoferus* | (*Oospora lactis*) | |
| (*Torulopsis lipofera*) | *G. wallroth* | |
| *L. starkeyi* | (*Oïdium wallroth*) | |
| (Starkey's soil yeast) | (*Oospora wallroth*) | |
| Genus *Rhodotorula:* | Genus *Mucor* | |
| *R. glutinis* | | |
| (*R. gracilis*) | Genus *Penicillium:* | |
| | *P. javanicum* | |
| Genus *Trichosporon:* | *P. lilacinum* | |
| *T. pullulans* | *P. spinulosum* | |
| (*Endomyces vernalis*) | *P. soppi* | |
| (*Endomycopsis vernalis*) | | |

* The names in parentheses are former ones.

used, the fat contents of the dried microbes, and their fat coefficients, are presented in Table 30.

**Fat Production by Rhodotorula glutinis.**[1] This yeast is of particular interest because of its high fat content (up to 63 per cent on a dry basis); high fat coefficient (grams of fat produced from 100 g. of sugar); ability to grow readily in submerged culture, forming fat rapidly; and ease of recovery from the propagation medium.

The production of fat by *R. glutinis* has been studied by Enebo et al. (1944, 1946), Pan et al. (1949), Nielsen et al. (1949), Nielsen and Nilsson (1950), Lundin (1950), Nielsen and Rojowski (1950), Sandegren et al. (1950), Steinberg and Ordal (1954), and others.

*Carbohydrate Sources.* Although *R. glutinis* is able to utilize a number of sugars as carbon sources, the best ones for fat production are invert

---

[1] This yeast has been described under the name *R. gracilis* in most reports.

TABLE 30. SOME DATA CONCERNING FAT PRODUCTION BY YEASTS

| Yeast | Raw material | Fat content, % dry weight | Fat coefficient | Reference |
|---|---|---|---|---|
| *andida reukaufii* .... | ........... | 25.3 | ...... | *Branntweinwirtschaft*, **3**: 65 (1949). |
| *ipomyces lipoferus* .. | Glucose | 50–63 | ...... | *Biochem. Jour.*, **38**: 480 (1944). |
| | Molasses | ........ | ...... | *Advances in Enzymol.*, **8**: 299 (1948). |
| *. starkeyi* .......... | Glucose | 50–63 | 12–13 | *Wallerstein Labs. Communs.*, **9**(27): 160 (1946). |
| *hodotorula glutinis* .. | Glucose | 35.4 | 9.8 | *Svensk. Kemi Tidsskr.*, **55**: 41 (1943).<br>*Advances in Enzymol.*, **9**: 653 (1949). |
| *. glutinis* .......... | Invert sugar | 50–60 | 16–18 | *Arch. Biochem.*, **11**: 383 (1946). |
| | Molasses | 20.4–52.5 | 16–18 | *Arch. Biochem.*, **23**: 419 (1949). |
| | Glucose | 59.0 | 18.4 | *Arch. Biochem.*, **23**: 419 (1949). |
| | Xylose | ........ | 7.2–9.5 | *Arch. Biochem.*, **25**: 316 (1950). |
| *ichosporon pullulans* ............... | Molasses | 45 | 10–12 | *Chem. Ztg.*, **61**: 689, 723, 744 (1937). |
| | Sulfite liquor | ........ | ...... | *Zeit. angew. Chem.*, **35**: 110 (1922).<br>Germant Patents 305091 and 306365, 1920. |

gar, glucose, fructose, mannose, sucrose, and those of blackstrap molas-
s (Pan et al., 1949). Xylose, while less suitable, may be used satisfac-
rily by this yeast after adaptation (Nielsen and Nilsson, 1950).

Ordinarily sugar concentrations of 4 to 8 per cent are used for fat
rmation. Increasing the sugar content of the medium to about 20 per
nt has no effect on the fat content of the yeast, but higher concentra-
ns result in reduced fat content (Steinberg and Ordal, 1954).

*Nitrogen Sources.* A deficiency of nitrogen induces fat formation
her than protein formation in the yeast. A concentration of 1 g. of
nmonium sulfate per liter was found to be optimum by Enebo et al.
944). Pan and associates (1949) observed that an insufficient supply
ammonium sulfate and potassium acid phosphate in a blackstrap-
lasses medium resulted in incomplete utilization of the sugar. When

these two ingredients were each used in the amount of 0.5 g. per liter, there was complete sugar utilization and a high fat coefficient. A concentration of 1 g. per liter of ammonium sulfate alone gave comparable results.

*Effect of Sulfur and Iron Deficiencies.* A deficiency of sulfur, or of iron, in the medium, as well as a deficiency of nitrogen, may induce increased fat formation (Nielsen and Rojowski, 1950).

*Effect of pH.* The rate of fat formation varies linearly with the pH between pH 3 and 8.5 and increases from 2.1 to 3.1 g. of fat per 100 g. of nonfat yeast per hour (Steinberg and Ordal, 1954).

The pH of the molasses medium used by Pan and associates (1949) was usually 4.6 to 4.8.

*Temperature.* The optimum temperature for fat production is about 28°C. The fat rate (grams of fat per 100 g. of nonfat dry yeast per hour) at 28°C. (2.79) is over twice that at 22°C. (1.09), according to Steinberg and Ordal (1954).

*Example of Production.* The medium used by Enebo et al. (1944) in most of their experiments had the following composition:

| Constituent | | |
|---|---|---|
| Invert sugar | 40.0 | g./liter |
| $(NH_4)_2SO_4$ | 1.0 | g./liter |
| $K_2HPO_4$ | 1.0 | g./liter |
| $MgSO_4 \cdot 7H_2O$ | 1.0 | g./liter |
| NaCl | 0.5 | g./liter |
| $CaCl_2 \cdot 6H_2O$ | 0.5 | g./liter |
| $FeCl_3 \cdot 6H_2O$ | 0.005 | g./liter |
| Beer wort (20 per cent) | 25 ml. | |
| pH (with $H_2SO_4$) to | 4.5 | |

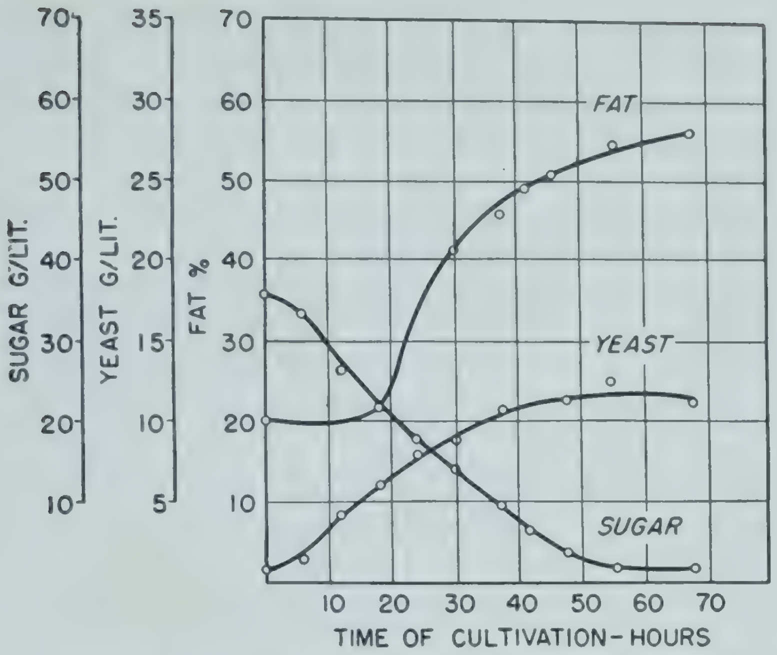
The yeast was grown in a cylinder of acid-resistant steel, which was 25 cm. in diameter and 225 cm. high and which was provided with an aerating device at the bottom and a rotating foam suppressor.

The temperature of incubation was 27 to 29°C.

OBSERVATIONS. In one typical experiment, the time of cultivation was 68 hr.; the volume of nutrient solution, 47 liters; the volume of the seed culture, 5.5 liters containing 46 g. of yeast on a dry basis; the newly formed yeast (dry substances), 547 g.; the added sugar, 1,880 g.; the yield of yeast, 29.1 per cent; the content of crude fat in dry-yeast substance, 56.6 per cent; the newly formed fat, 326 g.; the fat coefficient, 17.3; the degree of reproduction, 12.9; the number of generations, 3.69; the generation time, 18.4 hr.; the nitrogen content of the yeast, 2.10 per cent; and the ash content of the yeast, 3.5 per cent.

Figure 16 shows the relationship between sugar content, quantity of yeast, and fat content, as functions of time.

*The Amino Acids of* R. glutinis. The amino acid contents of *R. glutinis* when grown in media containing 7.6 per cent nitrogen and 2.8 per cent nitrogen, respectively, were compared by Nielsen et al. (1949). It was found that about the same amino acids were produced by the yeasts in each medium. There was no $\beta$-alanine present in the protein-poor yeast,



IG. 16. Sugar content, quantity of yeast, and fat content as functions of time. *Courtesy of L. Enebo, L. G. Anderson, and H. Lundin, Arch. Biochem.*, 11(3): 383 1946).]

nd this yeast contained more α-alanine and glutamic acid. The protein-ich yeast contained more tryptophan.

**Fat Production by Lipomyces lipoferus.** Starkey (1946) has de-cribed fat production by a yeast isolated from the soil, which is now esignated as *L. lipoferus.* He found that the lipid content of the cells was rgest when the yeasts were grown on a nitrogen-deficient carbohydrate edium. Large amounts of lipid were produced in an aerated solution at contained 3 per cent glucose, 0.05 per cent $(NH_4)_2SO_4$, and 0.01 per nt yeast extract. The conversion of glucose to lipid varied from 10 to per cent; the lipid content of cells (on a dry basis), from 50 to 63 r cent.

**Fat Production by Trichosporon pullulans.** Considerable research as undertaken by Lindner and his associates at the Institut für Gärungs-werbe of Berlin at the time of World War I in an effort to produce fat om an organism which was then known as *Endomyces vernalis*, still later *Endomycopsis vernalis*, and now as *T. pullulans* (Lodder and Kreger-an Rij, 1952).

*T. pullulans* grows as a mat or skin over the surface of a liquid medium. xygen appears to be essential for fat production by this microbe, yet it as not been possible to cultivate it under submerged-culture conditions. Several carbohydrates are assimilated but not fermented. Since fat orage is sought, this is a desirable feature of the organism. Molasses, llulose waste, hydrolyzed wood, and other media containing an assim-ible source of carbohydrate may be used as raw materials for fat oduction.

Waste sulfite liquor, fortified with nitrogenous substances and the cessary salts, is a favorable raw material. Nitrogen-containing substances may include yeast water, ammonium salts, urea, urine, molasses slop, extracts of grains, or other products. Potassium chloride, primary potassium phosphate, and magnesium sulfate may be used as salts.

An abundance of a suitable carbohydrate is essential for the maximum production of fat.

The optimum temperature for growth is 15 to 20°C., although a temperature as low as 10°C. may be used.

In the production of fat by *T. pullulans* (Fink, Haehn, and Hoerburger, 1937), two phases may be recognized in the incubation period: the phase during which the principal growth of the organism occurs (2 to 3 days under optimum conditions) and the phase of principal fat formation, which generally requires 6 to 8 days in addition. The phase of growth has been designated as the "protein generation" by Lindner. During this phase, the organism may be satisfactorily used for inoculation purposes. For growth the organism may be cultivated in a medium rich in nitrogen but poor in carbohydrates, but for fat production ("fat generation") a medium rich in a carbohydrate is essential. Thus is illustrated the difference between the optimum conditions for growth and optimum conditions for the production of a desirable end product.

Though many efforts were made to find satisfactory methods for producing fat from *T. pullulans,* only two methods were carried out on a large scale: the floor process and the pan process.

1. *The Floor Process.* In this process, inert materials such as chopped straw or coarse sawdust were washed, impregnated with the nutrient medium, and sterilized. The impregnated inert material was spread in thin loose heaps over the floor and inoculated with a fine suspension of *T. pullulans.* The heaps were turned several times a day and occasionally sprayed with water. At 11°C., fat production was completed in approximately 12 days; at 20°C., in 8 to 10 days. The fungus was dried at a temperature of not above 50°C.

The data (Lindner) in Table 31 are taken from a test in which the inert material was impregnated with a 25 per cent molasses solution and inoculated with *T. pullulans.*

TABLE 31. DATA ON FAT PRODUCTION BY *Trichosporon pullulans*

| | |
|---|---|
| Amount of sugar used | 12.5 kg. |
| Sugar used in $CO_2$ production | 4.16 kg. |
| Sugar left for fat formation | 8.34 kg. |
| Theoretical yield of fat | 3.33 kg. |
| Actual yield of fat | 0.95 kg. |
| Percentage of theoretical yield | 28.5 |
| Yield on basis of sugar used | 7.6 per cent |

The amount of sugar converted to fat by this process was small. Furthermore, difficulties were encountered in separating the fungus from the inert material, frequent attention was necessary during growth of the organism, and infection, especially with *Torula,* was prevalent. There was, however, adequate surface exposure.

2. *The Pan Process.* In this process, which was carried out on a

rge scale, the organism was grown in large, flat pans containing a shal- ow layer (1 to 2 cm. in depth) of a sterilized nutrient solution of sugar.

In order to conserve space and to facilitate handling, the pans were laced one above the other in frames. No covers were placed over the ans.

When the mat, or growth, on the surface of the solution in the pan ecame well developed, a large part of the culture solution was carefully in off and replaced with fresh nutrient sugar solution. After the maxi- um fat formation (usually in 7 to 8 days), the spent culture solution as drawn off and the mat carefully washed by repeated underlayerings ith water. The mats thus obtained, rich in fat, were used as a paste. uch paste was designated, for example, as "Evernal" or "Myceta." The rotein content of such pastes was valuable.

In this process sugar utilization was nearly complete, but the neces- ary use of much hand labor increased the costs, while infection by yeasts, olds, or bacteria was a very serious matter in some cases.

*Recovery of Fat.* Fat may be obtained from *T. pullulans* by one of everal methods: by chemical means, by extraction with ether, and by an utolytic process. In the chemical method the fungus mat is treated ith warm dilute hydrochloric acid to decompose the cells. The fat, btained as neutral fat, may be used for food. The efficiency of the rocess is high.

The cells are ground with sand to disintegrate them before extraction ith ether. Fat so obtained is used industrially. The efficiency of this ocess is not high.

Self-digestion is permitted at approximately 50°C. for 2 to 3 days in e autolytic process. The fat is recovered from the autolyzate.

*Preservation of the Fat.* The fat keeps well if oxygen is completely cluded.

**Early Attempts at Fat Production with Yeasts.** Nägeli and Loew 878) first proved that carbohydrates could be transformed to fat by ast.

Fat production is a normal function of yeast cells, but the rate of its rmation and the quantity of it stored may be increased through the e of certain optimum conditions. Factors of importance in fat pro- ction are the use of a medium rich in carbohydrate, oxygenation, and osphates.

Oxygenation of a medium is essential if greater than normal quantities fat are desired. Nägeli in 1878 showed that the fat content of yeasts d molds could be increased from 5 to 12 per cent when they were own in well-oxygenated media containing an abundance of carbo- drates and a scarcity of nitrogen.

The addition of alkaline phosphates ($Na_2HPO_4$ and $KH_2PO_4$) to a ll-oxygenated medium containing 4 per cent glucose caused increased storage in a suspension of brewery yeast (Smedley-MacLean and ffert, 1924).

Usually the rate of fat formation was greatest at the beginning of the eriment, diminishing later. When the concentration of sugar was increased, the amount of phosphate absorbed by the yeast cells was likewise augmented.

It has been demonstrated that oxygenating a solution containing a suitable sugar increases the amount of fat stored in the suspended yeast. If yeast containing reserve carbohydrate is suspended in oxygenated water, part of the carbohydrate is converted into fat (Smedley-MacLean, 1936).

Although investigations have been carried out using oxygenated solutions of various simple carbon compounds, thus far only ethyl alcohol and sodium acetate, 2-carbon compounds, have produced significant increases in the amount of fat stored by the suspended yeast (Smedley-MacLean and Hoffert, 1926). These compounds must be used in relatively dilute solution (a 0.5 to 0.6 per cent concentration of ethyl alcohol is satisfactory), because increasing the concentration of either above a low maximum leads to poor results.

The rate at which oxygen is supplied to solutions of ethyl alcohol and sodium acetate determined chiefly the amounts of lipids stored in yeast. When a solution of these compounds is not oxygenated, the storage of fat and carbohydrate is not increased.

Alcohol vapor, in the presence of oxygen, leads to a deposition of lipoid material in the cells of brewery yeast and also in *T. pullulans,* according to the researches of Lindner and Unger (1919). Yeast (Frohberg type), when grown in agar in a chamber containing the vapors of ethyl alcohol and oxygen, showed definite increases in fat content (Halden, 1934). By keeping the moisture content of the yeast low through the use of a drying atmosphere, the highest yields of fat were obtained in the presence of alcohol vapor. Substitution of ethyl alcohol with propyl alcohol in control experiments yielded negative results.

The following table indicates the average increases in the fat content of samples of yeast incubated in oxygenated water and in oxygenated solutions of $N/14$ acetates (MacLeod and Smedley-MacLean, 1938).

TABLE 32. AVERAGE INCREASES IN FAT CONTENT OF YEAST IN VARIOUS SOLUTIONS

| | Percentage Increase in Fat Content |
|---|---|
| Water | 41 |
| Potassium acetate | 180 |
| Sodium acetate | 160 |
| Magnesium acetate | 118 |
| Calcium acetate | 100 |

When phosphate is added to a 0.6 per cent solution of sodium acetate, there is no increase in the quantity of fat produced from the acetate.

Fat is produced from sodium lactate and sodium pyruvate in oxygenated solutions containing yeast, according to Smedley-MacLean and coworkers. Shaking the solutions of these compounds increased the quantity of fat and carbohydrate stored. Lactic acid, or lactate, usually yields carbon dioxide and small amounts of volatile acids in addition to e stored products. Pyruvate is not so effective as lactate in forming orage compounds.

No increases in the lipid content of yeast, not equally well obtained oxygenating a suspension of yeast in water, were observed by oxygen-ing solutions of the sodium salts of citric, crotonic, fumaric, gluconic, vulic, maleic, or succinic acids; acetoin; 2:3-butyleneglycol; or methyl-hyl ketone.

The addition of calicum or magnesium ions to an oxygenated solution glucose significantly decreased the quantity of lipids usually stored in east. Likewise, the addition of these ions to oxygenated solutions of cetates caused a decrease in the amount of lipid material ordinarily rmed from the acetates.

### Some References concerning Bakers' Yeast

nonymous: How Bakers' Yeast Is Made, *Food Eng.*, **23**(5): 122–125 (1951).

alls, A. K., and J. B. Brown: Studies in Yeast Metabolism, I. Introduction, *Jour. Biol. Chem.*, **62**: 789–821 (1925); II. Carbon Dioxide and Alcohol, *Jour. Biol. Chem.*, **62**: 823–836 (1925).

rockmann, M. C., and T. J. B. Stier: Steady State Fermentation by Yeast in a Growth Medium, *Jour. Cellular Comp. Physiol.*, **29**(1): 1–14 (1947).

urton, L. V.: *Food Inds.*, **15**(1): 66 (1943).

ampbell, L. A.: Yeast, Its Production and Uses, *Can. Chem. Processing*, October, 1953.

astor, J. G. B., and T. J. B. Stier: Apparatus for Continuous Yeast Culture, *Science*, **106**(2741): 43 (1947).

Becze, G., and A. J. Liebmann: Aeration in the Production of Compressed Yeast, *Ind. Eng. Chem.*, **36**: 882 (1944).

weson, E. W.: Yeast from Wood, *Chem. Inds.*, **38**: 573 (1936).

ustel, I. C., and H. Humfeld: A New Laboratory Fermenter for Yeast Production Investigations, *Jour. Bact.*, **52**(2): 229–235 (1946).

scher, A. M.: Yeasts and Factors Determining Its Vitamin Potency, *Brewers Dig.*, **13**(10): 37 (1938).

ey, C. N.: History and Development of the Yeast Industry, *Ind. Eng. Chem.*, **22**: 1154 (1930).

——: History of the Development of Active Dry Yeast, in "Yeast, Its Characteristics, Growth, and Function in Baked Products, Quartermaster Food and Container Institute for the Armed Forces," Chicago, January, 1957, pp. 7–33.

——, G. W. Kirby, and A. Schultz: Yeast Physiology, Manufacture and Uses, *Ind. Eng. Chem.*, **28**: 879 (1936).

ipper, C. H.: Five Keys to Growing Nutritional Yeast, *Food Inds.*, **22**: 1375–1380 (1950).

odder, J.: Die anaskosporogenen Hefen, Erste Hälfte, *Verhandel. Koninkl. Ned. Wetenschap. Afdel. Natuurk.*, Sec. II, **32**: 1–256 (1934).

acDonough, J. V., and T. C. Haffenreffer, Jr.: *Wallerstein Labs. Communs.*, **7**(20): 39 (1944).

enzinsky, G.: On Factors Influencing the Metabolism and Growth of *Saccharomyces cerevisiae* (Top Yeast) under Aerobic Conditions, *Arkiv Kemi*, **2**(1): 1–94 (1950).

erritt, P. P.: Role of Active Dry Yeast in Commercial Bread Manufacture, in "Yeast, Its Characteristics, Growth, and Function in Baked Products, Quartermaster Food and Container Institute for the Armed Forces," Chicago, January, 1957, pp. 94–99.

rdsiek, F. W.: Bakers Yeast—Unique Product, *Food Inds.*, **23**(2): 101–108 (1951).

veek, P. L., W. H. Peterson, and C. A. Elvehjem: Effect of Growth Conditions on Yield and Vitamin $B_1$ of Yeast, *Ind. Eng. Chem.*, **29**: 536 (1937).

———, ———, and ———: Factors Affecting the Vitamin $B_1$ Content of Yeast, *Ind. Eng. Chem.*, **30**: 802 (1938).

Peterson, W. H., J. T. Skinner, and F. M. Strong: "Elements of Food Biochemistry," Prentice-Hall, Inc., Englewood Cliffs, N.J., 1943.

Stephany, C. D., and H. W. Von Loesecke: "Selected References on Yeast," War Food Administration, Washington, January, 1945.

Unger, E. D., W. H. Stark, R. E. Scalf, and P. Kolachov: Continuous Aerobic Process for Distiller's Yeast, *Ind. Eng. Chem.*, **34**: 1402–1405 (1942).

Wachtel, M.: *Münch. med. Wochschr.*, **76**: 1513 (1929).

Walter, F. G.: "The Manufacture of Compressed Yeast," 2d ed., Chapman & Hall, Ltd., London, 1953.

White, J.: A Critical Survey of Methods for the Determination of Yeast Quantity, *Am. Brewer*, **80**(8): 40 (1947).

———: "Yeast Technology," John Wiley & Sons, Inc., New York, 1954.

———: Influence of Temperature on Yeast Growth and Fermentation and on Yeast Cell Water Relations, *Am. Brewer*, April, 1955, pp. 41–45.

——— and D. J. Munns: Nutrilites and the Production of Pressed Yeast, *Jour. Inst. Brewing*, **56**(3): 194–202 (1950).

Yamada, K., J. Takahashi, T. Yosimura, and T. Yano: Oxygen Absorption Coefficient as a Factor of Yeast Propagation, *Jour. Agr. Chem. Soc. Japan*, **30**: 22–26 (1956).

### Some Patents

Bennett, W. G.: Manufacture of Yeast, U.S. Patent 2,059,980, Nov. 3, 1936.

Bertel, R.: Manufacture of Dry Yeast, U.S. Patent 1,859,250, May 17, 1932.

Browne, H. H.: Manufacture of Yeast, U.S. Patent 2,166,339, July 18, 1939.

Buhrig, W. H., A. Schultz, and C. N. Frey: Compressed Yeast Product, U.S. Patent 1,970,275, Aug. 14, 1934.

Darànyi, S. C.: Manufacture of Yeast, U.S. Patent 2,035,048. Mar. 24, 1936.

Epstein, A. K.: Process of Growing Yeast, U.S. Patent 1,858,488, May 17, 1932.

Freshel, C. P.: Process for the Treatment of Yeast and the Product Produced Thereby, U.S. Patent 1,981,225, Nov. 20, 1934.

Heijkenskjöld, O. W.: Method of Manufacturing Yeast, U.S. Patent 1,881,557, Oct. 11, 1932.

Irvin, R. R., and M. W. Mead, Jr.: Process for the Production of Yeast, U.S. Patent 2,183,570, Dec. 19, 1939.

Jansen, S.: Manufacture of Yeast, U.S. Patent 2,056,576, Oct. 6, 1936.

Jellinck, E.: Process of Producing Yeast without Alcohol, U.S. Patent 1,920,395, Aug. 1, 1933.

Kirby, G. W., and C. N. Frey: Manufacture of Yeast, U.S. Patent 2,029,572, Feb. 4, 1936.

Kitzmeyer, E. L.: Process of Manufacturing Yeast, U.S. Patent 2,097,292, Oct. 26, 1937.

Lockey, J.: Aeration Apparatus, U.S. Patent 2,151,126, Mar. 21, 1939.

Meyer, E. A.: Manufacture of Yeast, U.S. Patent 1,938,081, Dec. 5, 1933.

Mizobata, K.: Method of Preparing Dried Yeast, U.S. Patent 1,934,941, Nov. 14, 1933.

Moskovits, M.: Manufacture of Yeast of High Enzymatic Activity, U.S. Patent 1,962,831, June 12, 1934.

Nilsson, M., and N. S. Harrison: Process for Producing Yeast, U.S. Patent 1,449,127, Mar. 20, 1923.

Pollak, A.: Manufacture of Yeast, U.S. Patent 2,094,023, Sept. 28, 1937.

Reich, G. T.: Art of Purifying Yeast, U.S. Patent 2,031,668, Feb. 25, 1936.

Riley, H.: Process for Treating Yeast, U.S. Patent 1,909,011, May 16, 1933.

———: Preserved and Dried Yeast, U.S. Patent 1,991,629, Feb. 19, 1935.

Sak, S.: Method of Controlling Yeast Propagation, U.S. Patent 1,884,272, Oct. 25, 1932.

———: Yeast Propagation Controlling Method, U.S. Patent 1,891,841, Dec. 20, 1932.

Schultz, A.: Manufacture of Yeast, U.S. Patent 2,029,592, Feb. 4, 1936.

———: Manufacture of Yeast, U.S. Patent 2,079,634, Mar. 11, 1937.

——— and C. N. Frey: Manufacture of Yeast, U.S. Patent 1,893,152, Jan. 3, 1933.

——— and F. R. Swift: Manufacture of Yeast, U.S. Patent 2,717,837, Sept. 13, 1955.

haver, A., and A. S. Schultz: Manufacture of Yeast, U.S. Patent 1,910,265, May 23, 1933.

teibelt, W.: U.S. Patent 2,085,709 (1937).

homas, C. A., and C. A. Hochwalt: Fermentation Process and Product, U.S. Patent 2,138,434, Nov. 29, 1938.

an Thiel, E.: Apparatus for the Manufacture of Yeast, U.S. Patent 2,043,940, June 9, 1936.

ogelbusch, W.: Apparatus and Process for Aerating Wort in Yeast Production, U.S. Patent 2,121,458, June 21, 1938.

hite, J. R.: Yeast Composition and Process of Making the Same, U.S. Patent 1,974,937, Sept. 25, 1934.

———: Dry Yeast and Method of Preparing the Same, U.S. Patent 1,974,938, Sept. 25, 1934.

illiams, R. J.: Yeast Culture in Bread Making and Yeast Propagation, U.S. Patent 2,147,762, Feb. 21, 1939.

———: Method of Producing Yeast, U.S. Patent 2,174,543, Oct. 3, 1939.

roten, J. F.: Process for Manufacturing Yeast, U.S. Patent 1,917,283, July 11, 1933.

## Some References concerning Food and Fodder Yeasts

garwal, P. N., K. Singh, P. S. King, and W. H. Peterson: Yields and Vitamin Content of Food Yeasts Grown on Different Kinds of Molasses, *Arch. Biochem.*, **14**(1 and 2): 105–115 (1947).

alls, A. K.: Yeast Production from Wood Sugars by the Bergins Process, *FIAT Final Report* 493, Jan. 3, 1946.

ickel, A.: Zur Kenntnis des ernährungsphysiologischen Wertes des Hefeeiweisses, *Biochem. Zeit.*, **310**: 355–377 (1942).

lock, R. J., and D. Bolling: The Amino Acids Yielded by Various Yeasts after Hydrolysis of the Fat-Free Material—A Comparative Investigation, *Arch. Biochem.*, **7**: 313–321 (1945).

ünger, H. et al.: *Landwirtsch. Vers.-Sta.*, **121**: 193 (1934).

utschek, G.: *Chem. Ztg.*, **12**: 230 (1944).

arter, H. E., and G. E. Phillips: The Nutritive Value of Yeast Proteins, *Federation Proc.*, **3**: 123 (1944).

olonial Food Yeast Limited: "Food Yeast—A Venture in Practical Nutrition," Brook House, London, 1944.

ill, D. B.: Manufacture of Torula Food Yeast, Zellstoffabrik Waldhof, *Report* 201, Department of Commerce, Office of the Publication Board.

irr, K.: Über den Wert der Wuchshefen für die menschliche Ernahrung. II. Mitteilung: Über die biologische Brauchbarkeit der Bergin-Holzzuckertrockenhefe, *Biochem. Zeit.*, **312**: 233–251 (1942).

——— und O. v. Soden: Über den Wert der Wuchshefen für die menschliche Ernahrung. I. Mitteilung: Aminosäuregehalt von Bergin-Holzzuckertrockenhefe, *Biochem. Zeit.*, **309**: 329–336 (1941).

——— und ———: IV. Über die Lipoide der Wuchshefen, *Biochem. Zeit.*, **312**: 263–276 (1942)R.

unn, C. G.: Food Yeast, *Wallerstein Labs., Communs.*, **15**(48): 61–79 (1952).

———: The Industrial Utilization of Yeasts, *Econ. Botany*, **12**(2): 145–163 (1958).

ingerling, G., und F. Honcamp: *Landwirtsch. Vers.-Sta.*, **117**: 263 (1933).

nk, H., und F. Just: Zur Biochemie der *Torula utilis*. II. Mitteilung: Über die zusammensetzung der Hefesubstanz, insbesondere des Hefeeiweissses, *Biochem. Zeit.*, **300**: 84–88 (1938).

——— und ———: Zur Biochemie der *Torula utilis*. IV. Mitteilung: Der Gesamtschwefel, Glutathion- und Cystingehalt verschiedener Hefen, *Biochem. Zeit.*, **303**: 234–241 (1939).

Fink, H., und F. Just: Zur Biochemie der *Torula utilis.* VII. Mitteilung: Zugleich Ergänzung zu der Mitteilung von K. Dirr und O. v. Soden "Über den Wert Wuchshefen für die menschliche Ernahrung. I. Mitteilung. Aminosäuregehalt von Bergin-Holzzuckertrockenhefe, *Biochem. Zeit.,* **312**: 390–393 (1942).

Graham, V. E., D. L. Gibson, and H. W. Klemmer: II. Investigations with Small-scale Laboratory Fermenters, *Can. Jour. Technol.,* **31**: 92–97 (1953).

———, ———, ———, and J. M. Naylor: Increasing the Food Value of Whey by Yeast Fermentation. I. Preliminary Studies on the Suitability of Various Yeasts, *Can. Jour. Technol.,* **31**: 85–91 (1953).

———, ———, and W. C. Lawton: III. Pilot Plant Studies, *Can. Jour. Technol.,* **31**: 109–113 (1953).

Harris, E. E., M. L. Hannan, and R. R. Marquardt: Production of Fodder Yeast, *Paper Trade Jour.,* Nov. 27, 1947.

———, J. F. Saeman, R. R. Marquardt, M. L. Hannan, and S. C. Rogers: Fodder Yeast from Wood Hydrolyzates and Still Residues, *Ind. Eng. Chem.,* **40**(7): 1220–1223 (1948).

Harris, G. C. M.: Food Yeast: The Mycologists' Contribution to Post War Nutrition, *Rev. Appl. Mycol.,* **25**: 132 (1945).

Hock, A.: Über den biologischen Ergänzungswert verschiedener Nahrungsproteine, I Mitteilung: Der Ersatz von tierischem Eiweiss durch Hefeeiweiss, *Biochem. Zeit.,* **311**: 385–401 (1942).

——— und H. Fink: *Zeit. physiol. Chem.,* **279**: 187 (1943).

Holderby, J. M.: *U.S. Dept. Commerce, OTS, PB* 34807 (1946).

Inskeep, G. C., A. J. Wiley, J. M. Holderby, and L. P. Hughes: Food Yeast from Sulfite Liquor, *Ind. Eng. Chem.,* **43**: 1702–1711 (1951).

Just, F., und H. Fink: Zur Biochemie der *Torula utilis.* III. Mitteilung: Vergleichende Untersuchungen über den Phosphatgehalt von *Torula utilis* und Bierhefe, *Biochem. Zeit.,* **303**: 1–9 (1939).

Klose, A. A., and H. L. Fevold: Nutritional Value of Yeast Protein to the Rat and the Chick, *Jour. Nutrition,* **29**: 421–430 (1945).

Kurth, E. F.: Yeasts from Wood Sugar Stillage, *Ind. Eng. Chem.,* **38**(2): 204–207 (1946).

——— and V. H. Cheldelin: Feeding Yeasts from Wood Sugar Stillage, *Ind. Eng. Chem.,* **38**: 617–619 (1946).

Lechner, R.: Über die Verhefung der Pentosen, *Angew. Chem.,* **53**: 163–167 (1940)

———: Über die Ausnutzung der Pentosen bei der biologischen Eiweissynthese. V Mitteilung: Züchtung von *Torula utilis* in Xylose und in Galaktose, *Biochem. Zeit.,* **304**: 84–89 (1940).

———: Über die Ausnutzung der Pentosen bei der biologischen Eiweissynthese. VI. Mitteilung: Züchtung von *Torula utilis* in Arabinose, Rhamnose und Glucuronsäure, *Biochem. Zeit.,* **306**: 218–223 (1940).

Lewis, J. C.: Relationship of Iron Nutrition to the Synthesis of Vitamins by *Torulopsis utilis, Arch. Biochem.,* **4**(2): 217–228 (1944).

———, J. J. Stubbs, and W. M. Noble: Vitamin Synthesis by Torula Yeast, *Arch. Biochem.,* **4**(3): 389–401 (1944).

Locke, E. J.: Yeast Production in Germany from Sulfite Waste Liquors, *Pulp & Paper Ind.,* **20**(1): 20–26 (1946).

Lodder, J., and N. J. W. Kreger-van Rij: "The Yeasts, a Taxonomic Study," North Holland Publishing Co., Amsterdam, 1952.

Markham, A. E., H. D. Agar, and J. L. McCarthy: Continuous Laboratory Propagation of Sulfite Waste Liquor, Yeast, *Tappi,* **34**(3): 131–140 (1951).

Mukherjee, J. K.: A Review of the Present Knowledge on the Production of Food Yeast, unpublished report at the Massachusetts Institute of Technology, Cambridge, Mass., January, 1947.

Nolte, A. J., H. W. von Loesecke, and G. N. Pulley: Feed Yeast and Industrial Alcohol from Citrus Press Juice, *Ind. Eng. Chem.,* **34**: 670 (1942).

Northeastern Wood Utilization Council: Wood Yeast for Animal Feed, *Bull.* 12, New Haven, Conn., November, 1946.

Pavcek, P. L.: Wood-sugar Yeast Manufacture, *PB Report* 4292.

eters, R. A.: Food Yeast: A Summary of Its Nutritive Value, *Med. Research Council (Brit.), War Memo.* 16 (1945).

terson, W. H., J. F. Snell, and W. C. Frazier: Fodder Yeast from Wood Sugar, *Ind. Eng. Chem.*, 37: 30–35 (1945).

eiser, C. O.: Torula Yeast from Potato Starch Wastes, *Jour. Agr. Food Chem.*, 2(2): 70–74 (1954).

osenquist, O.: How Sweden Produces Yeast for Nutritional Purposes, *Food Inds.*, 16(6): 443–444, 487 (1944).

eman, J. F., E. G. Locke, and G. K. Dickerman: The Production of Wood Sugar in Germany and Its Conversion to Yeast and Alcohol, *FIAT Final Report* 499, Nov. 14, 1945.

heunert, A., und K. H. Wagner: Weitere Untersuchungen über den Vitamin $B_1$- und $B_2$-Gehalt von Holzzuckertrockenhefen, *Biochem. Zeit.*, 303: 329–334 (1939).

——, ——, H. Fink, und J. Krebs: Die Synthese von Vitamin $B_1$ and $B_2$ (Komplex) durch die Hefe *Torula utilis*, *Biochem. Zeit.*, 302: 1–11 (1939).

koog, F.: Food Yeast Production and Utilization in Germany, *PB Report* 2041.

erber, E.: Studies in the Metabolism of Growing *Torulopsis utilis* under Aerobic Conditions, *Arkiv Kemi, Mineral. Geol.*, 21(3): 1–136 (1945).

ephenson, W., C. Penton, and V. Korenchevsky: *Brit. Med. Jour.*, II: 839 (1941).

haysen, A. C.: Value of Micro-organisms in Nutrition (Food Yeast), *Nature*, 151(3832): 406–408 (1943).

——: Production of Food Yeast, *Food*, May, 1945, pp. 116–119.

—— and M. Morris: Preparation of a Giant Strain of *Torulopsis utilis*, *Nature*, 152(3862): 526–528 (1943).

silensis, E., and L. R. Hedrick: Growth of Food Yeasts in Olive Residue Hydrolyzate, *Food Technol.*, 4(3): 107–109 (1952).

eldhuis, M. K., and W. O. Gordon: Experiments on Production of Feed Yeast from Citrus Press Juice, *Proc. Florida State Hort. Soc.*, 1947, p. 32.

on Soden, O., und K. Dirr: III. Verdaulichkeit in vitro von verschiedenen Hefen im Vergleich zu anderen Eiweisstragen der menschlichen Ernahrung, *Biochem. Zeit.*, 312: 252–262 (1942).

alker, R. D., Jr., and R. A. Morgen: Protein Feed from Sulphite Waste Liquor, *Paper Trade Jour.*, 123(6): 43–47 (Aug. 8, 1946).

ilson, F. A.: Yeast in Nutrition, *Intern. Sugar Jour.*, 46: 154–156 (1944).

## Some References on Brewers' Yeast

unlop, S. G.: Dried Yeast in Army Rations, *Modern Brewery Age*, 33(3): 24 (1945).

irbrother, T. H.: Food Yeast, *Food Manuf.*, 19(9): 309 (1944).

ffenreffer, T. C., Jr.: Practical Application of Yeast Conservation and Processing, *Brewers Bull.*, 36(87): 4 (1943). Refer also to *Food Inds.*, 15(11): 66 (1943).

hm, J. B.: Microbiological Examination and Evaluation of Brewers' Yeast, *Wallerstein Labs. Communs.*, 6(19): 191–197 (1943).

bel, R. V., P. J. F. Weber, and E. Singruen: Functions and Properties of Vitamins in Waste Brewers' Yeast, *Modern Brewery Age*, 26(5): 44 (1941).

——, ——, and ——: Production Phases of Waste Brewers' Yeast, *Modern Brewery Age*, 26(6): 46 (1941).

——, ——, and ——: Spray Drying Brewers' Yeast, *Modern Brewing Age*, 27(1): 49 (1942).

——, ——, and ——: Drum Drying Brewers' Yeast, *Modern Brewery Age*, 27(2): 74 (1942).

## Some References on Fat Production by Yeasts

lls, A. K.: Production of Fat by *Oïdium lactis*, *FIAT Final Report* 371, Oct. 10, 1945.

ijerinck, M. W.: Levures chromogenes. nouvelle réaction biologique du fer, *Arch. néerl. physiol.*, 2: 609 (1918).

l, H. B.: "The Biochemistry of Lipids," John Wiley & Sons, Inc., New York, 1937.

Dunn, C. G.: Food Yeast, *Wallerstein Labs. Communs.*, **15**(48): 61–79 (1952).

Enebo, L., L. G. Anderson, and H. Lundin: Microbiological Fat Synthesis by Means of Rhodotorula Yeast, *Arch. Biochem.*, **11**(3): 383–395 (1946).

———, M. Elander, F. Berg, H. Lundin, R. Nilsson, and K. Myrbäch: *Iva*, **6**: 1 (1944).

Fink, H., H. Haehn, und W. Hoerburger: Über die Versuche zur Fettgewinnung mittels Mikroorganismen mit besonderer Berücksichtigung der Arbeiten des Institut für Gärungsgewerbe, *Chem. Ztg.*, **61**: 689–693, 723–726, 744–747 (1937).

———, G. Haeseler, und M. Schmidt: *Zeit. Spiritusind.*, **60**: 74, 76, 81 (1937).

Geffers, H.: *Arch. Mikrobiol.*, **8**: 66–98 (1937).

Guilliermond, A.: "The Yeasts," translated and revised by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

Haehn, H., und W. Kinttof: Über den chemischen Mechanismus bei der Fettbildung in der lebenden Zelle, *Ber.*, **56**: 439 (1923).

Halden, H.: *Biochem. Zeit.*, **225**: 249 (1934).

Hesse, A.: Industrial Biosyntheses. Part I. Fats, *Advances in Enzymol.*, **9**: 663–704 (1949).

Hinsberg, O., und E. Roos: Über einige Bestandteile der Hefe, *Zeit. physiol. Chem.*, **38**: 1 (1903).

——— und ———: Nachtrag zu der Abhandlung über einige Bestandteile der Hefe, *Zeit. physiol. Chem.*, **42**: 189 (1904).

Hoogerheide, J. C.: Yeast Lipids and Their Biological Synthesis, in "Yeasts in Feeding, Proceedings of the Symposium," Milwaukee, November, 1948, pp. 20–25.

Kleinzeller, A.: Fat Formation in *Torulopsis lipofera*, *Biochem. Jour.*, **38**: 480–492 (1944).

Lindner, P.: Das Problem der biologischen Fettbildung und Fettgewinnung, *Zeit. angew. Chem.*, **35**: 110 (1922).

——— und Unger: *Wochschr. Brau.*, **36**: 188 (1919).

Lundin, H.: Fat Synthesis by Microorganisms and Its Possible Applications in Industry, *Jour. Inst. Brewing*, **56**: 17–28 (1950).

MacLeod, L. D., and I. Smedley-MacLean: Yeast Metabolism, *Biochem. Jour.*, **32**: 1571 (1938).

Nadson, G. A., and H. G. Konokotine: "Fat Yeast," *Endomyces vernalis*, as a Source of Fat for Foods and Technical Purposes, *Wochschr. Brau.*, **41**: 249 (1924); *Chem. Abstracts*, **19**: 2219 (1925).

Nägeli, C., und O. Loew: Über die chemische Zusammensetzung der Hefe, *Ann.*, **193**: 322 (1878).

Nielsen, N., and N. G. Nilsson: Investigations on Respiration, Growth, and Fat-production of *Rhodotorula gracilis* when Cultivated in Media Containing Different Carbohydrates, *Arch. Biochem.*, **25**: 316–322 (1950).

——— and P. Rojowski: On the Sulfur Metabolism of *Rhodotorula gracilis*. I. The Importance of Sulfur and Iron for the Formation of Protein and Fat, *Acta Chem. Scand.*, **4**: 1309–1310 (1950).

———, E. Sandegren, and L. Ljundahl: Amino Acids in *Rhodotorula gracilis* Rennerfelt, *Nature*, **164**: 1055 (1949).

Pan, S. C., A. A. Andreasen, and P. Kolachov: Factors Influencing Fat Synthesis by *Rhodotorula gracilis*, *Arch. Biochem.*, **23**: 419–433 (1949).

Rippel, A.: Biologische Fettgewinnung durch Hefen im Lüftungsverfahren, *Naturwissenschaften*, **31**: 248 (1943).

Sandegren, E., D. Ekstrom, and N. Nielsen: On the Sulfur Metabolism of *Rhodotorula gracilis*. II. The Ratio between SH and SS Groups, *Acta Chem. Scand.*, **4**: 1311–1313 (1950).

Smedley-MacLean, I.: The Conditions Influencing the Formation of Fat by the Yeast Cell, *Biochem. Jour.*, **16**: 370 (1922).

———: The Biochemical Synthesis of Fat from Carbohydrate, *Ergeb. Enzymforsch.*, **5**: 285 (1936).

——— and D. Hoffert: The Carbohydrate and Fat Metabolism of Yeast, *Biochem. Jour.*, **17**: 720 (1923).

——— and ———: The Carbohydrate and Fat Metabolism of Yeast. II. The Influence of Phosphates on the Storage of Fat and Carbohydrate in the Cell, *Biochem. Jour.*, **18**: 1273 (1924).

—— and ———: The Carbohydrate and Fat Metabolism of Yeast. III. Nature of the Intermediate Stages, *Biochem. Jour.*, **20**: 343 (1926).

—— and E. M. Thomas: The Nature of Yeast Fat, *Dept. Sci. Ind. Research (Brit.) Food Invest., Mem.* 9, 1920.

tarkey, R. L.: Lipid Production by a Soil Yeast, *Jour. Bact.*, **51**: 33–50 (1946).

teinberg, M. P., and Z. N. Ordal: Effect of Fermentation Variables on Rate of Fat Formation by *Rhodotorula gracilis*, *Jour. Agr. Food Chem.*, **2**: 873–877 (1954).

White, A. G. C., and C. H. Werkman: Fat Synthesis in Yeast, *Arch. Biochem.*, **17**: 475–482 (1948).

Wieland, H., und F. Wille: Weitere Versuche über die Dehydrierung von Alkohol durch Hefe. Über den Mechanismus der Oxydationsvorgänge, XLI, *Ann.*, **515**: 260 (1935).

## CHAPTER 4

# THE PRODUCTION OF INDUSTRIAL ALCOHOL BY FERMENTATION

One of the most important and best-known industrial fermentations is that in which ethyl alcohol is produced from sugars by yeasts. The chemical manufacturer, the brewer, the distiller, the baker, the vinegar manufacturer, the scientist, the housewife, and many others depend in one way or another on the ability of the yeast to convert sugars to alcohol, carbon dioxide, and other end products. Since low-priced and waste carbohydrate materials may be used in the manufacture of ethyl alcohol, the fermentation process has not only great present but also vast potential value.

**Definitions.** Ethyl alcohol ($CH_3CH_2OH$), or ethanol, may be referred to by other names. For example, the alcohol may be named to indicate the source of raw material from which it is manufactured or to indicate the general purpose for which it is to be used. Grain alcohols are alcohols made from grains, such as corn, wheat, or rice. The term "grain alcohol" is sometimes used to signify ethyl alcohol in contrast to methyl alcohol ($CH_3OH$), or methanol, which is manufactured by the destructive distillation of wood, by synthesis, or by other means. Molasses alcohol is alcohol produced from sugar-cane molasses. Industrial alcohol is ethyl alcohol used for industrial purposes. Under this term is included power or fuel alcohol, i.e., alcohol used in combination with gasoline or other motor fuels.

The units of measurement commonly used in reporting ethyl alcohol production are the proof gallon, wine gallon, tax gallon, and barrel. The standard proof gallon is a wine gallon containing 50 per cent by volume (100 proof) of ethyl alcohol. A wine gallon is equal to a standard United States gallon and contains 231 cu. in. A tax gallon is equivalent to a proof gallon for spirits of a concentration of 100 proof or more and is equivalent to a wine gallon for spirits of a concentration less than 100 proof. A barrel contains 31 wine gal.

Proof is twice the per cent in volume of ethyl alcohol. For example, 95 per cent ethyl alcohol by volume is 190 proof.

**Production Statistics.** The quantity of ethyl alcohol produced by 11 leading states and by the United States during the fiscal years 1936, 1946, and 1956 is shown in Table 33.

During the fiscal year ending June 30, 1956, the leading states for ethanol production from ethyl sulfate were Texas, Louisiana, West Vir-

## TABLE 33. ETHYL ALCOHOL PRODUCTION BY LEADING STATES DURING THE FISCAL YEARS 1936, 1946, AND 1956 *

(Thousands of proof gallons)

| State | 1936 | 1946 | 1956 |
|---|---|---|---|
| Texas | ...... | 25,596 | 128,914 |
| Louisiana | 28,557 | 71,956 | 105,323 |
| Pennsylvania | 45,714 | 56,789 | 74,995 |
| Illinois | 8,983 | 1,414 | 67,567 |
| West Virginia | 15,287 | 45,429 | 66,181 |
| Indiana | 16,202 | 23,208 | 25,732 |
| Washington | ...... | 2,837 | 6,551 |
| California | 4,896 | 7,674 | 3,833 |
| Kansas | 20 | 3,245 | 2,717 |
| New Jersey | 31,137 | 21,392 | |
| Maryland | 28,168 | 16,695 | |
| Total, United States | 196,126 | 329,387 | 485,484 |

* U.S. Department of the Treasury, Internal Revenue Service.

## TABLE 34. ETHYL ALCOHOL PRODUCTION BY KINDS OF MATERIALS *

| Kind of material | Ethyl alcohol produced, per cent of total | | |
|---|---|---|---|
| | 1936 | 1946 | 1956 |
| hyl sulfate | 16.06 | 36.07 | 59.34 |
| lasses | 76.15 | 24.64 | 25.54 |
| hylene gas | .... | .... | 9.80 |
| ducts used in redistillation | .... | 6.70 | 2.12 |
| fite liquors | .... | 0.80 | 1.32 |
| ain and grain products | 7.04 | 26.69 | 1.20 |
| llulose pulp and chemical mixtures; rude alcohol mixtures | .... | 0.55 | 0.52 |
| ey | .... | 0.04 | 0.09 |
| eapple juice | 0.32 | 0.07 | 0.07 |
| elose | .... | .... | † |
| it wine | .... | .... | † |
| mented liquor | 0.03 | | |
| rn sirup | 0.03 | | |
| tatoes and potato products | .... | 1.07 | |
| rus waste concentrate | .... | 0.01 | |
| rn-sugar by-products | .... | † | |
| er mixtures | 0.37 ‡ | 0.36 | |
| otal | 100.00 | 100.00 | 100.00 |

U.S. Department of the Treasury, Internal Revenue Service. *Annual Reports of Commissioner*, for the fiscal years ended June 30, 1936, 1946, and 1956, respec-ly.

Less than 0.01 per cent.

Includes sulfite liquor, molasses, and hydrol.

ginia, Illinois, and Indiana; from molasses, Pennsylvania, Louisiana, California, Puerto Rico, and Illinois; from corn, Illinois; from wheat, Kansas (most) and Illinois; from sorghum grain, Kansas; from rye, Illinois and Kansas; and from malt, Illinois and Kansas.

**Raw Materials.** Ethyl alcohol may be produced from any fermentable sugar by yeasts under suitable conditions. Since starches and certain other carbohydrates may be hydrolyzed to fermentable sugars by biological or chemical means, there are available many possible sources of sugar.

1. *Types.* Raw materials may be classified into three principal types: (*a*) the saccharine materials, such as sugar cane, sugar beets, molasses, and fruit juices; (*b*) the starchy materials, which include the cereals (corn, malt, barley, oats, rye, wheat, rice, grain, sorghum, and the like),

TABLE 35. PRODUCTION OF ETHYL ALCOHOL BY KINDS OF MATERIALS USED, FISCAL YEAR, 1956 *

| Kind of material | Quantity used | | Ethyl alcohol produced | |
|---|---|---|---|---|
| | | | Proof gal. | % of total |
| Ethyl sulfate | 232,537,504 | gal. | 294,487,566 | 59.34 |
| Molasses | 104,991,918 | gal. | 126,753,404 | 25.54 |
| Ethylene gas | 110,037,766 | lb. | 48,657,779 | 9.80 |
| Products used in redistillation | 10,796,237 | proof gal. | 10,506,318 | 2.12 |
| Sulfite liquors | 289,075,200 | gal. | 6,551,148 | 1.32 |
| Grain and grain products,† total | 69,615,211 | lb. | 5,940,589 | 1.20 |
| Corn | 32,907,590 | lb. | | |
| Malt | 2,044,766 | lb. | | |
| Wheat | 18,192,060 | lb. | | |
| Sorghum grain | 15,150,065 | lb. | | |
| Rye | 1,320,730 | lb. | | |
| Cellulose pulp and chemical and crude alcohol mixtures: | | | | |
| Cellulose pulp and chemical mixtures | 3,158,381 | gal. | | |
| Crude alcohol mixtures | 1,327,842 | gal. | 2,574,262 | 0.52 |
| Whey ‡ | 13,030,015 | gal. | 463,500 | 0.09 |
| Pineapple juice | 4,446,365 | gal. | 339,270 | 0.07 |
| Cerelose | 35,600 | lb. | 4,836 | § |
| Fruit wine | 5,508 | gal. | 1,035 | § |
| Total | | | 496,280,127 ¶ | 100.00 |

* U.S. Department of the Treasury, Internal Revenue Service, Alcohol and Tobacco Tax Division, *Statistics Relating to the Alcohol and Tobacco Industries for th Fiscal Year Ended June* 30, 1956, *Publication* 67, 1956.

† In addition 1,792,900 gal. and 20,050 lb. of enzyme extract was used in the production of ethyl alcohol from grain and grain products.

‡ In addition 20,590 gal. of yeast cream was used in the production of ethyl alcohol from whey.

§ Less than 0.01 per cent.

¶ Represents gross production. Net production—that is, the gross production minus products used in redistillation—was 485,483,890 proof gal.

tatoes, sweet potatoes, Jerusalem artichokes (girasol), manioca meal, d other substances; and (*c*) cellulosic materials, such as wood and aste sulfite liquor.

2. *Principal Raw Materials Utilized.* In the United States, molasses d grain are the principal carbohydrate materials used in the production fermentation ethanol. Because of the relatively high cost of molasses d the favorable costs of ethyl sulfate and ethylene gas, a larger and rger proportion of the total production is being made by chemical synesis. Table 34 indicates the trend in the utilization of raw materials. ble 35 supplies data on kinds of materials.

Various types of raw materials are used in different countries. In ermany, for example, potatoes are used extensively; in France, sugar ets. Sweden manufactures much industrial alcohol from sulfite pulp. Italy, sugar beets, molasses, grapes, and other substances are used.

## PROCESSES OF MANUFACTURE

The process used in the manufacture of ethyl alcohol by fermentation pends on the nature of the raw material. Saccharine materials usually quire little or no special preliminary treatment other than dilution and ay be fermented directly after certain customary adjustments have been ade in the mash. But starchy and cellulosic materials must be hydrozed to fermentable sugars before they can be utilized by the yeast.

In each of the processes, success depends on the efficiency of prelimiry treatment, if any; the use of an optimum concentration of sugar, an timum pH, and an optimum temperature; the addition of nutrient stances to the mash, if it is deficient in any essential constituent; ibition of bacterial growth; the use of a vigorous strain of yeast with h alcoholic tolerance and also capable of producing large yields of ohol; the maintenance of anaerobic conditions during the fermentation oper; and the prompt distillation of the fermented mash.

1. **Ethyl Alcohol from Molasses.** *a. Outline of Process.* The molas- mash is adjusted to the desired sugar concentration and temperature the addition of water and to the desired pH by the addition of a asured quantity of acid. A yeast "starter" is mixed with the mash in fermentation tank, which is usually covered, in one of several manrs. Streams of the adjusted mash and the starter flowing simultanesly into the fermentor may be caused to converge on a baffle board ated in the upper part of the tank. The mash and starter become ll mixed as they spatter and fall to the bottom of the tank. An altere method is to add the starter after the mash has been placed in tank and effect mixing of the two by compressed air from lines ated at the bottom of the tank. Another method would be to use ddles.

The fermentation rapidly becomes vigorous with the evolution of ge quantities of carbon dioxide. In the modern plant, this gas is colted, purified, and used for the manufacture of dry ice or for other purposes. Within 50 hr., or less, the fermentation is usually complete. The fermented molasses, referred to as "beer," is distilled in a continuous still to separate the alcohol and other volatile constituents from the mash. The alcohol is purified by means of rectifying columns and then stored in a bonded warehouse or denatured.

*b. The Process in Detail.* (1) TYPE OF YEAST. Certain types of yeasts are desirable, namely, those which are able to produce and tolerate high concentrations of alcohol and which possess uniform and stable characteristics. Strains of *Saccharomyces cerevisiae* are commonly used, but other yeasts, such as *S. anamensis* and *Schizosaccharomyces pombe,* may be employed under certain conditions.

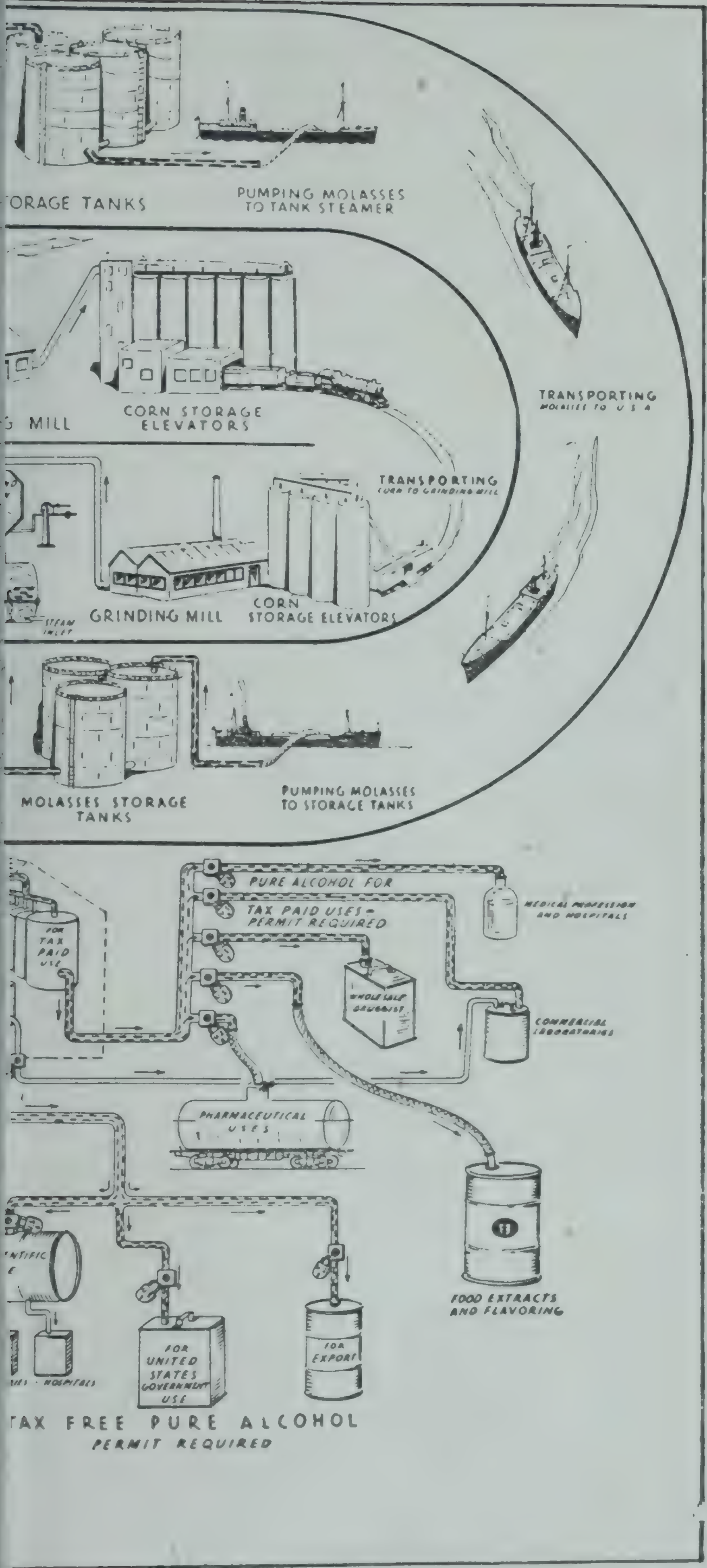
(2) PREPARATION OF STARTER. Having selected the yeast for the fermentation and having isolated it in pure culture, a starter is then prepared. A starter of large volume is required to "pitch" (inoculate) the main mash, which frequently may have a magnitude of several thousand gallons. By means of aseptic technique, a tube containing about 10 ml. of sterile wort is inoculated from a pure culture of the yeast, which may be mantained on malt agar media. After incubation for a suitable period of time at a temperature of 25°C. (77°F.) to 30°C. (86°F.), the optimum for yeast growth, the culture in the tube may be used to inoculate a flask containing approximately 200 ml. of sterile mash. Following incubation, the contents of the flask may be used to seed a sterile mash of about 4-liter capacity. Up to this point in the preparation of the starter, the work is ordinarily carried out in the laboratory, where glass containers are used. The next mash inoculated is of semi-plant-scale size (10 to 40 gal.) and is located close to the fermentors. Usually at least one more proportionally larger mash (several hundred gallons) is inoculated and permitted to incubate. Then this fermenting mash, the starter, is either pumped or allowed to flow by gravity to the main mash. The addition of this enormous yeast culture to the mash constitutes "pitching."

Aeration is of benefit in preparing a starter, for the object is to secure an immense number of yeast cells.

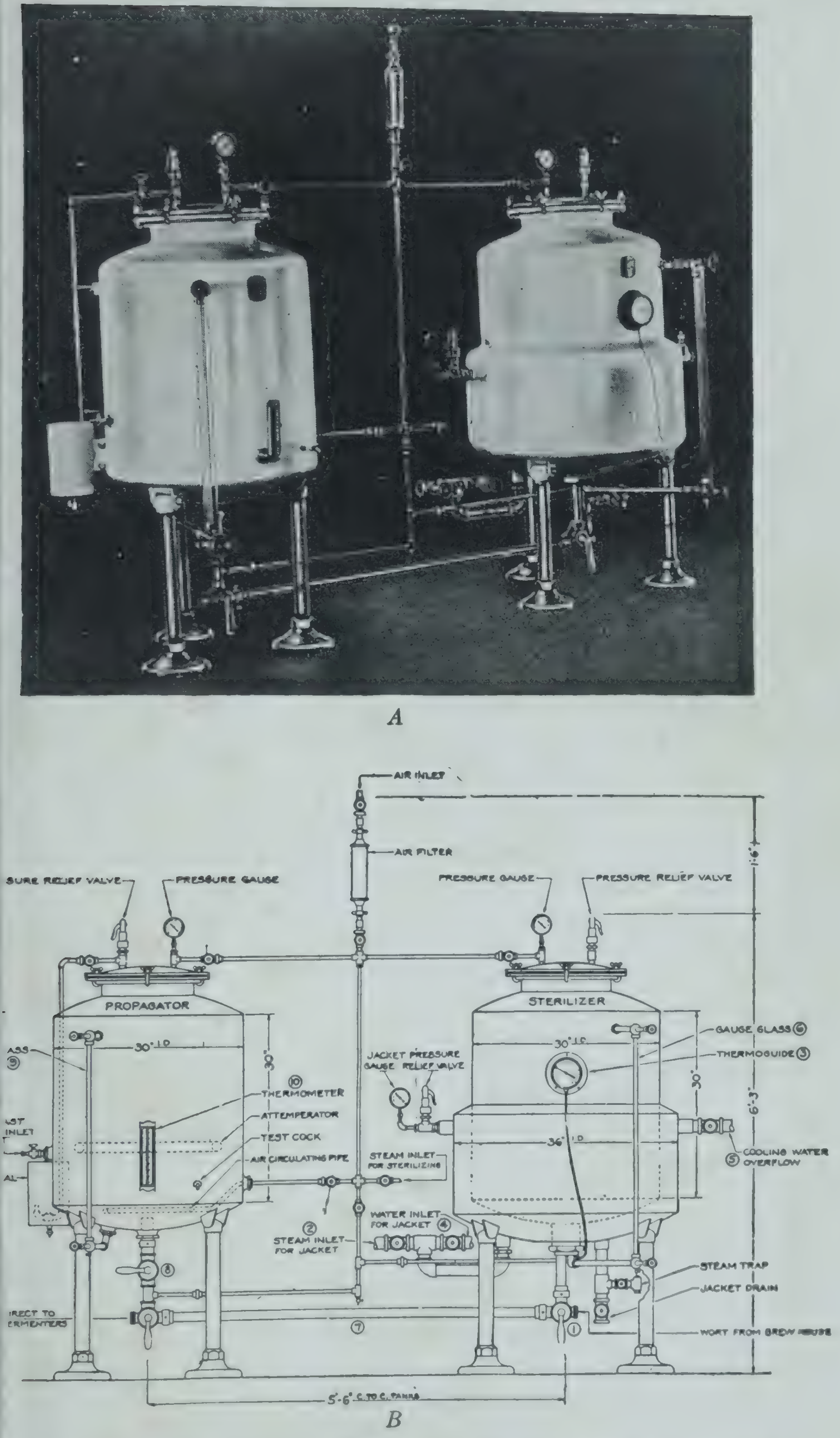
The Magné automatic system or other pure-culture systems (Fig. 18) for preparing the starter may be used instead of the method just outlined. In the Magné apparatus a stock of the pure culture is maintained in the upper drum of the apparatus. Mashes are inoculated from this pure culture as required, and one culture may be used over a considerable period of time before recharging with a pure culture derived from a single cell.

(3) THE MOLASSES. "Blackstrap" molasses is the principal source of industrial alcohol. This material is the sirup that is left after the recovery of crystalline sugar from the concentrated juice of sugar cane. It usually contains 48 to 55 per cent of sugars, mainly sucrose. A large part of the blackstrap molasses used in this country is imported from Cuba, although some comes from Puerto Rico.

During recent years, much "high-test" molasses has been used for ethanol manufacture. This so-called "molasses" is an evaporated sugar-cane juice that contains all the original sugar of the juice, but most


TORAGE TANKS
PUMPING MOLASSES TO TANK STEAMER
TRANSPORTING MOLASSES TO U S A
G MILL
CORN STORAGE ELEVATORS
TRANSPORTING CORN TO GRINDING MILL
STEAM INLET
GRINDING MILL
CORN STORAGE ELEVATORS
MOLASSES STORAGE TANKS
PUMPING MOLASSES TO STORAGE TANKS
FOR TAX PAID USE
PURE ALCOHOL FOR TAX PAID USES — PERMIT REQUIRED
MEDICAL PROFESSION AND HOSPITALS
WHOLESALE DRUGGIST
COMMERCIAL LABORATORIES
PHARMACEUTICAL USES
FOOD EXTRACTS AND FLAVORING
NTIFIC
IES · HOSPITALS
FOR UNITED STATES GOVERNMENT USE
FOR EXPORT
TAX FREE PURE ALCOHOL
PERMIT REQUIRED

*ional Distillers and Chemical Corp.)*


A
AIR INLET
AIR FILTER
SURE RELIEF VALVE
PRESSURE GAUGE
PRESSURE GAUGE
PRESSURE RELIEF VALVE
1'-6"
PROPAGATOR
STERILIZER
30" I.D.
30"
ASS
③
⑩
THERMOMETER
ATTEMPERATOR
TEST COCK
AIR CIRCULATING PIPE
AST
INLET
AL
JACKET PRESSURE GAUGE
RELIEF VALVE
STEAM INLET FOR STERILIZING
30" I.D.
36" I.D.
30"
6'-3"
GAUGE GLASS ⑥
THERMOGUIDE ③
⑤ COOLING WATER OVERFLOW
② STEAM INLET FOR JACKET
WATER INLET FOR JACKET ④
⑧
STEAM TRAP
JACKET DRAIN
IRECT TO
ERMENTERS
⑦
①
WORT FROM BREW HOUSE
5'-6" C. TO C. TANKS
B

IG. 18. Pure-yeast propagator. (*Courtesy of the Pfaudler Co., New York.*)

of it in an inverted form as a result of acid hydrolysis. Such molasses usually high in sugar, containing occasionally as much as 78 per cent.

(4) CONCENTRATION OF SUGAR. A sugar concentration of 10 to 1 per cent is usually satisfactory, although other concentrations are use When the concentration is too high it reacts adversely on the yeast, c the alcohol produced may inhibit the action of the yeast, with the consequence that the fermentation time is prolonged and some of the suga is not properly utilized. The use of too low a concentration of sugar i uneconomic as it may lead to a loss of valuable fermenting space. Furthermore a proportionally greater quantity of fermented wort must b distilled to produce the same amount of alcohol, thus adding to th expense of production.

A sugar concentration of approximately 12 per cent is frequently use The quantity of water required to reduce the molasses to the desire sugar content is calculated from available analytical data. For exampl 4 parts of water by weight would be added to 1 part of molasses, containing 60 per cent sugar, by weight, to reduce the sugar concentration t approximately 12 per cent.

The concentration of the sugar in a wort is usually determined b means of a Balling hydrometer, an instrument that gives the approximat percentage of sugar at 60°F., or 15.5°C.

(5) NUTRIENT SUBSTANCES. Although molasses generally contains mos of the nutrient substances required for fermentation, ammonium salts such as ammonium sulfate or phosphates, may be added to the mas to supply deficiencies in nitrogen or phosphorus.

(6) pH OF THE MASH. Fermentation proceeds satisfactorily when th mash has been adjusted to a pH of 4.0 to 4.5. This pH favors the yeas but is sufficiently low to inhibit the development of many types o bacteria. The manufacturer depends on the pH of the wort and th use of a large inoculum to take the place of sterilization, since it is costl and impracticable to sterilize large molasses mashes. Ordinarily th main mash is inoculated with a starter that represents 4 to 6 per cent o its volume, although the starter may represent from 2 to 25 per cent of th volume of the main mash under certain circumstances.

Sulfuric acid is commonly used to adjust the reaction of the mas although lactic acid is satisfactory. Lactic acid favors the developmen of yeast but inhibits the growth of the butyric acid bacteria, which ar detrimental to the yeast fermentation. As a substitute for the addition o acid, the mash may be inoculated with lactic acid bacteria, prior to th alcoholic fermentation.

(7) OXYGEN TENSION. Oxygen in large amounts is necessary in th early stages for the optimum reproduction of yeast cells but is no required for the production of alcohol. During the fermentation, carbo dioxide is evolved and anaerobic conditions are soon established.

(8) TEMPERATURE. The mash is pitched at a temperature of 60 t 80°F., usually 70 to 80°F., depending somewhat on the external temperature. During fermentation, the temperature of the mash rises. Th use of cooling coils or sprays on the outside of the tank helps to maintai

uitable temperature. At temperatures much above 80°F., alcohol porates rather rapidly. Bacterial growth is also favored.

9) TIME REQUIRED FOR FERMENTATION. A fermentation is usually com- e in 50 hr. or less, depending on the temperature, sugar concentration, other factors.

10) DISTILLATION. The fermented mash ("beer") is distilled to sepa- the ethyl alcohol and fusel oil from the other constituents of the sh. In case there is a shortage of fermentors and the "beer" cannot be distilled immediately, part of the "beer" is pumped to a storage k, known as a "beer well," where it is held until it can be distilled.

During distillation, fractions containing different concentrations of ohol ("wines") and slops are separated. The fractions containing roximately 60 to 90 per cent of ethyl alcohol are known as "high es." These fractions are concentrated to 95 per cent ethanol by her distillation or fractionation. The fractions low in alcohol, the v wines," are usually redistilled with new lots of "beer." The slops ordinarily discarded but may be used in a number of ways. Some- s the slops may be used as a substitute for some of the water in dilut- molasses for a new mash. The solids from slops may be concentrated heat-treatment and sold as a fertilizer constituent, for they contain ssium and phosphates in addition to other components. The slops be used as a core binder (in foundries) or as an adhesive for artificial e (briquette).

11) YIELD. The common yield from blackstrap molasses amounts to roximately 90 per cent of the theoretical, on the basis of the fer- table sugars.

2) FINAL TREATMENT. The 190-proof ethyl alcohol (95 per cent by me) may be further purified, dehydrated, or denatured as prescribed he Internal Revenue Service. Thus it is possible to purchase 95 per ethyl alcohol, with or without denaturant; c.p. (chemically pure) l alcohol of 96 per cent concentration; absolute alcohol, U.S.P.; and drous denatured ethyl alcohol (water-free alcohol).

3) FLOW SHEET. Schemes for the production of ethyl alcohol from toes, corn, and molasses are shown in Table 22.

*Continuous Processes.* A number of processes for the continuous uction of ethanol have been described, including laboratory processes Bilford and associates (1942), Owen (1948), Mariller et al. (1952), et al. (1952, 1953), and Borzani (1952, 1953, 1955, 1957). Patented esses for ethanol production include those of Kuffner and Kuffner 3), Dinaburg (1945*a*, *b*), Mattos (1948), Malchenko and Chistiakov 9), Borzani (1953), Keussler (1953), and others. The interested r is referred to these publications for detail and also to the book ed "Food for Thought," by Willkie and Kolachov.

**Ethyl Alcohol from Whey.** A successful method for producing alcohol from whey has been developed by Browne (1941) and by sa, Browne, and Whittier (1947), of the Division of Dairy Research ratories, Bureau of Dairy Industry, U.S. Department of Agriculture. e process, in brief, consists of heating the whey to boiling, adjusting the pH to 5, separating out the protein by filtration, cooling the clear whey to 34°C. (93°F.), adding 1 lb. of *Candida pseudotropicalis* per 120 gal. of whey, carrying out fermentation at 33 to 34°C. for 48 to 72 hr., separating out the yeast, and distilling the alcohol. A flow sheet of this process is shown in Fig. 19.

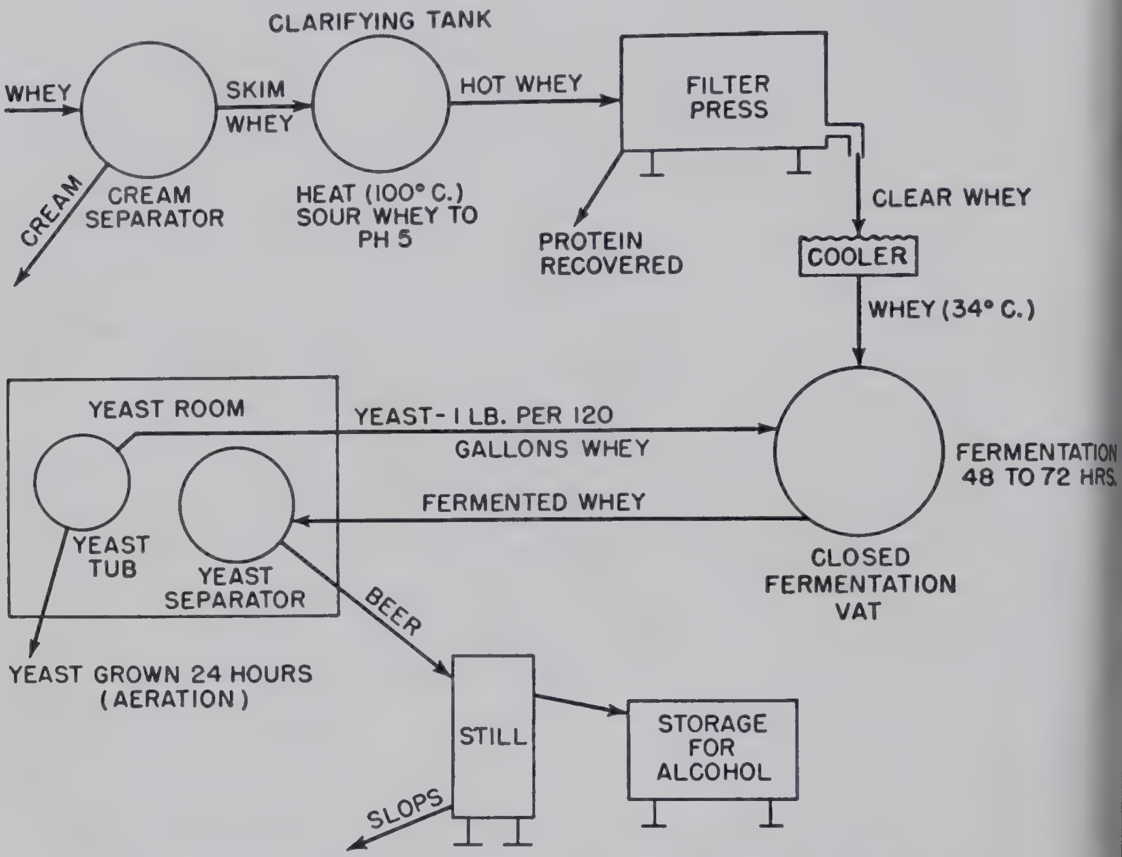
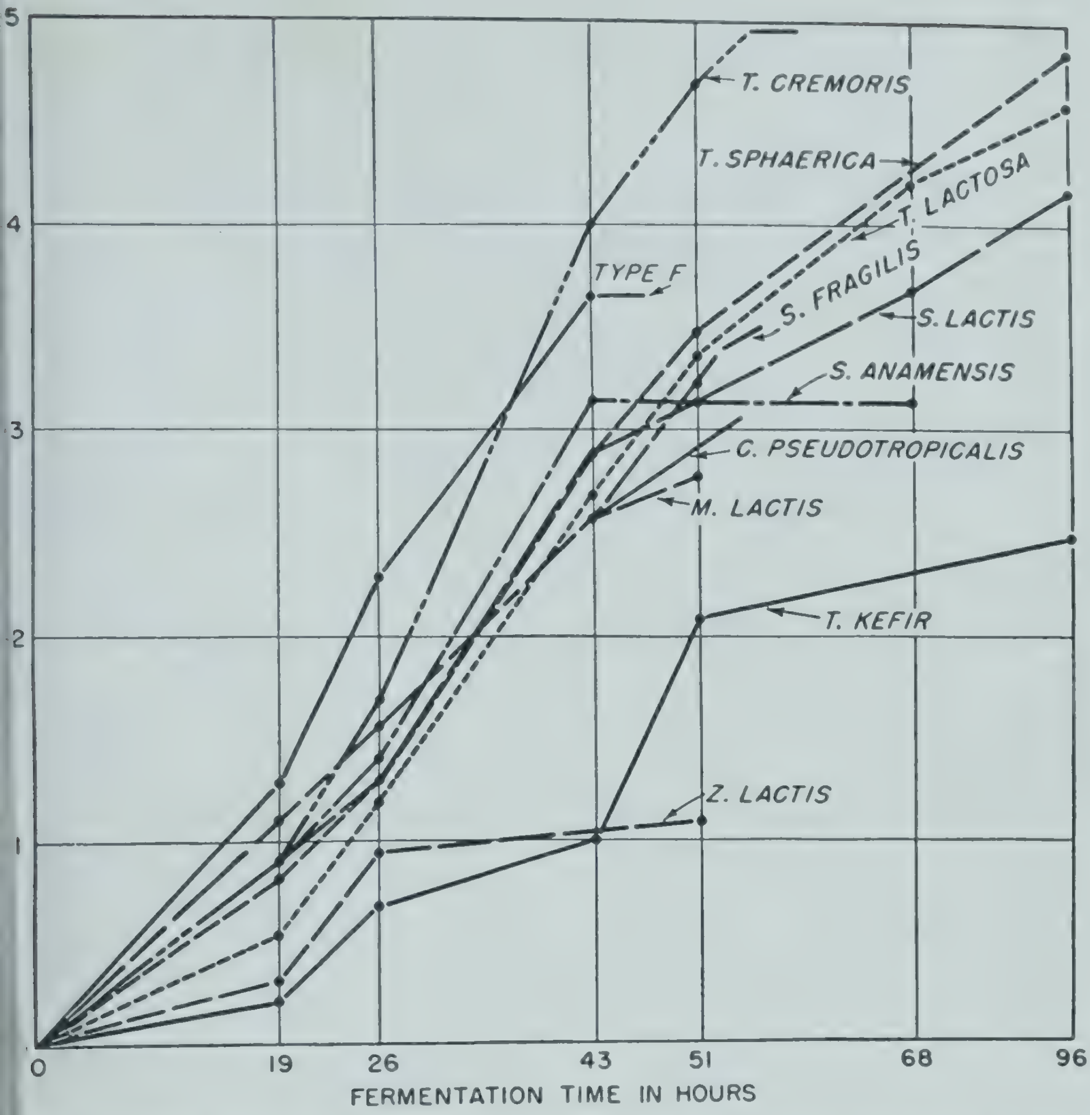

CLARIFYING TANK
WHEY
SKIM
WHEY
HOT WHEY
FILTER PRESS
CREAM
CREAM SEPARATOR
HEAT (100° C.) SOUR WHEY TO PH 5
PROTEIN RECOVERED
CLEAR WHEY
COOLER
WHEY (34° C.)
YEAST ROOM
YEAST - 1 LB. PER 120 GALLONS WHEY
FERMENTED WHEY
FERMENTATION 48 TO 72 HRS.
YEAST TUB
YEAST SEPARATOR
CLOSED FERMENTATION VAT
BEER
YEAST GROWN 24 HOURS (AERATION)
STILL
STORAGE FOR ALCOHOL
SLOPS

FIG. 19. Flow sheet for alcohol production from whey. [*Courtesy of M. Rogosa, H. H. Browne, and E. O. Whittier, Jour. Dairy Sci.,* 30(4): 263 (1947).]

Since the sugar of whey is lactose, it was necessary for Rogosa and his associates to examine a number of lactose-fermenting yeasts to find one that would ferment all of the lactose in a relatively short time. Their findings, in respect to whey containing 5 per cent of lactose, are illustrated in Fig. 20. It is evident from this chart that *Torula cremoris* (now named *C. pseudotropicalis*) was the most satisfactory yeast examined.

The maximum amount of yeast required for seeding was found to be 2 per cent of the weight of the lactose present in the whey before fermentation.

The use of a temperature of 33 to 34°C. is recommended by Rogosa and his coworkers, although fermentation is actually more rapid at 37°C. than at any other temperature in the range of 30 to 42°C. However, the loss of alcohol through evaporation is more rapid also at 37°C., and the slight savings in time accomplished through the use of this temperature are not warranted. Likewise in the fermentation of 150-gal. batches of whey started at 30°C. the temperature eventually reached a level of 33 to 34°C. at which level it remained during the most active part of the fermentation



20. Relative efficiency of lactose fermentation by lactose-fermenting yeasts. tesy of *M. Rogosa, H. H. Browne, and E. O. Whittier, Jour. Dairy Sci.*, 30(4): 1947).] (Refer to Lodder and Kreger-van Rij for new names of some of these s.)

arification may be carried out by heating the whey, adding sour or acid, and filtering off the protein. The initial pH of the clarified should lie between 4.8 and 5.2.

ogosa, Browne, and Whittier recommend that the pH of the whey be adjusted to a range of 4.7 to 5. Figure 21 shows pH changes rring during fermentations started at different initial pH levels.

elds of alcohol averaged 90.73 per cent on a laboratory basis, and as s 84 per cent under semiplant conditions.

-products of this fermentation are the whey protein and the slops, tter of which may be dried after the alcohol has been distilled in solution.

e need for the following basic equipment for plant operation has indicated by Rogosa and his associates on the basis of their research 150-gal. batches: a cream separator, a tank equipped with heat

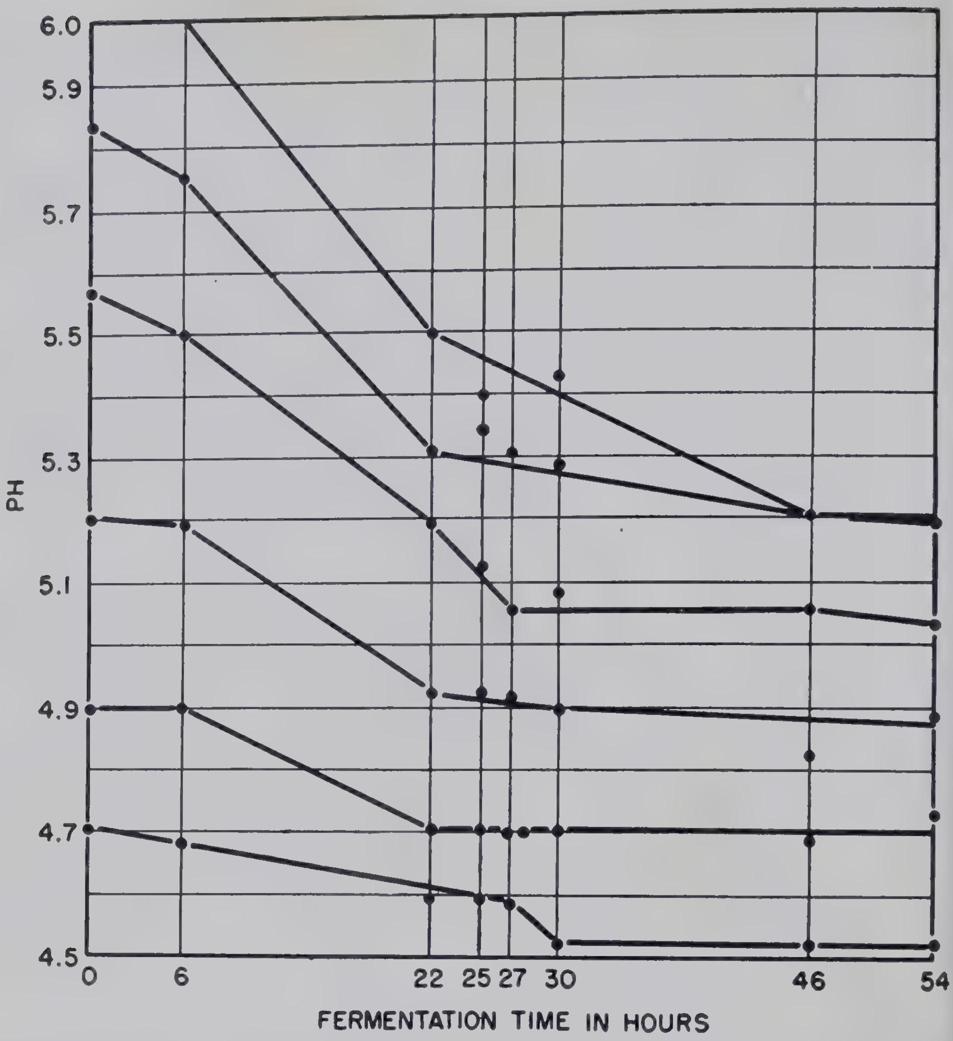


FIG. 21. Course of pH change during fermentation by *C. pseudotropicalis*. [*Court* *of M. Rogosa, H. H. Browne, and E. O. Whittier, Jour. Dairy Sci.,* 30(4): (1947).]

exchanger or liquid heater for heating the whey, a chemical feeder proportioner for handling acid or sour whey, a filter press, a cooler, clo fermentation vats (fermentors), yeast tubs and a yeast room, an air l and filter for supplying sterile air, a yeast separator, a still, a stor tank for the alcohol, facilities for concentrating and drying the slops desired), pumps, pipe lines, and a steam supply.

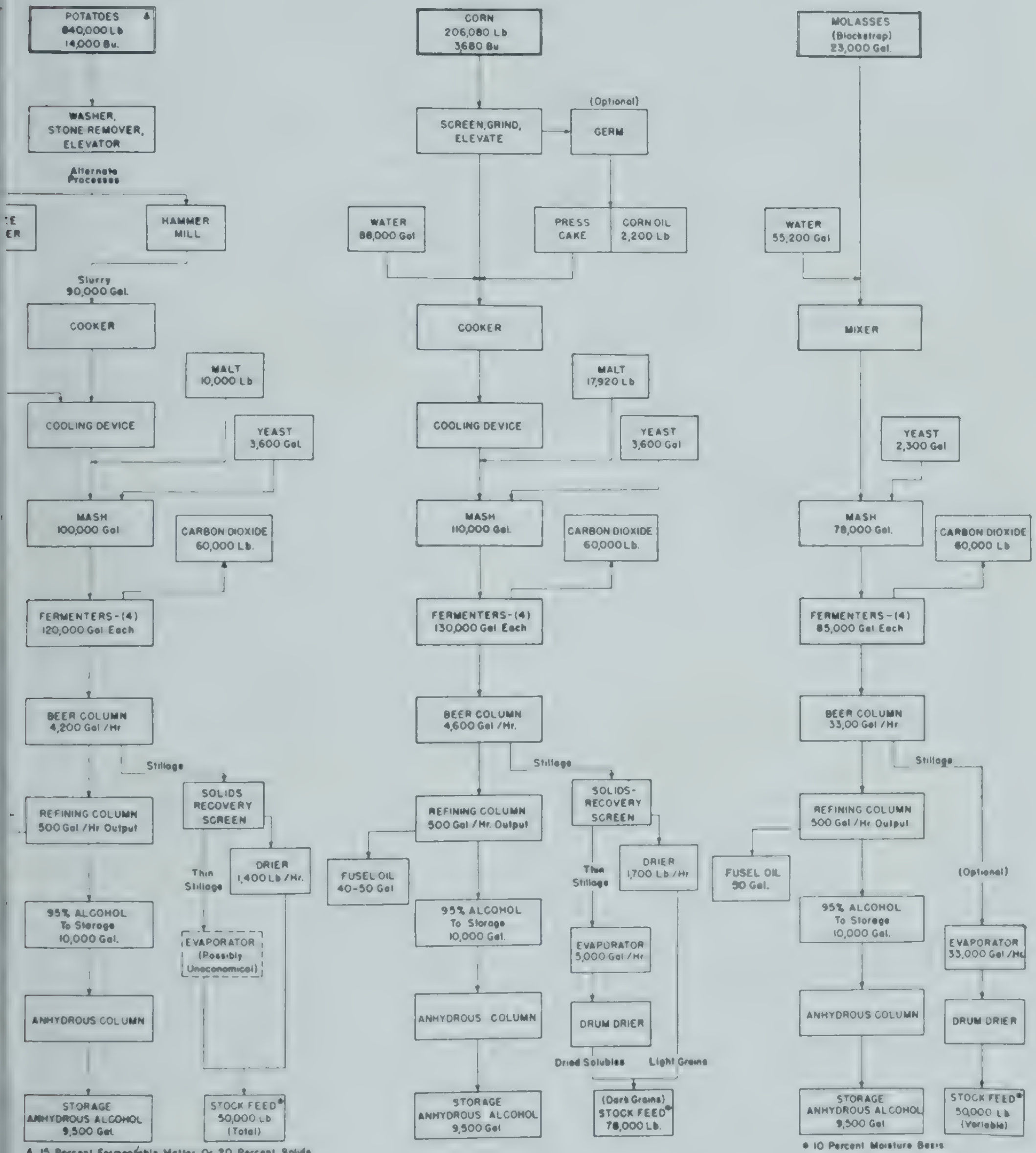
A partial list of manufacturers of some of the foregoing equipment supplied by the U.S. Department of Agriculture (BDIM-1011, M 1947).

**3. Ethyl Alcohol from Corn.** Normally corn is the chief grain sou of ethyl alcohol in this country.

*a. Preliminary Treatment.* Corn with or without the germs is grou mixed with water, and cooked with steam under pressure to gelatin the starch. The mash is then blown to a converter tub where additio water is added. After the temperature of the mash has been reduced about 140°F. (60°C.), barley malt is mixed with the mash. The enzym contained in the malt, convert much of the starch of the corn to a f table sugar (maltose) and also break down some of the protein mate- (Refer to the chapter on Brewing for details of the action of malt.) corn may be saccharified by the use of mold bran or acid or by her procedure (refer to Chap. 46).

he mash containing the saccharified starch is transferred to a fer- tor, adjusted to the proper sugar concentration by the addition of r, if necessary, cooled to a temperature of 65 to 80°F., and inoculated yeast.

*Flow Sheet.* A scheme for the production of ethanol from corn is n in Fig. 22.


POTATOES ▲
840,000 Lb
14,000 Bu.
WASHER, STONE REMOVER, ELEVATOR
Alternate Processes
E
ER
HAMMER MILL
Slurry 90,000 Gal.
COOKER
MALT 10,000 Lb
COOLING DEVICE
YEAST 3,600 Gal.
MASH 100,000 Gal
CARBON DIOXIDE 60,000 Lb.
FERMENTERS-(4) 120,000 Gal Each
BEER COLUMN 4,200 Gal /Hr
Stillage
SOLIDS RECOVERY SCREEN
REFINING COLUMN 500 Gal /Hr Output
Thin Stillage
DRIER 1,400 Lb /Hr.
95% ALCOHOL To Storage 10,000 Gal.
EVAPORATOR (Possibly Uneconomical)
ANHYDROUS COLUMN
STORAGE ANHYDROUS ALCOHOL 9,500 Gal
STOCK FEED* 50,000 Lb (Total)
CORN
206,080 Lb
3680 Bu
SCREEN, GRIND, ELEVATE
(Optional)
GERM
WATER 88,000 Gal
PRESS CAKE
CORN OIL 2,200 Lb
COOKER
MALT 17,920 Lb
COOLING DEVICE
YEAST 3,600 Gal
MASH 110,000 Gal.
CARBON DIOXIDE 60,000 Lb.
FERMENTERS-(4) 130,000 Gal Each
BEER COLUMN 4,600 Gal /Hr.
Stillage
SOLIDS-RECOVERY SCREEN
REFINING COLUMN 500 Gal /Hr. Output
FUSEL OIL 40-50 Gal
Thin Stillage
DRIER 1,700 Lb /Hr
95% ALCOHOL To Storage 10,000 Gal.
EVAPORATOR 5,000 Gal /Hr
ANHYDROUS COLUMN
DRUM DRIER
Dried Solubles
Light Grains
STORAGE ANHYDROUS ALCOHOL 9,500 Gal
(Dark Grains) STOCK FEED* 78,000 Lb.
MOLASSES
(Blackstrap)
23,000 Gal.
WATER 55,200 Gal
MIXER
YEAST 2,300 Gal
MASH 78,000 Gal.
CARBON DIOXIDE 60,000 Lb
FERMENTERS-(4) 85,000 Gal Each
BEER COLUMN 33,00 Gal /Hr
Stillage
REFINING COLUMN 500 Gal /Hr Output
FUSEL OIL 90 Gal.
(Optional)
95% ALCOHOL To Storage 10,000 Gal.
EVAPORATOR 33,000 Gal /Hr
ANHYDROUS COLUMN
DRUM DRIER
STORAGE ANHYDROUS ALCOHOL 9,500 Gal
STOCK FEED* 50,000 Lb (Variable)

▲ 15 Percent Fermentable Matter Or 20 Percent Solids

* 10 Percent Moisture Basis

2. Conventional alcohol process. The residues, as shown, will be increased by sence of yeast, yeast proteins, and unfermentable sugars. The figures actually re based merely on the unfermentable residues calculated from the various es of raw materials. (*Courtesy of P. Burke Jacobs and Harry P. Newton,* *ept. Agr., Bur. Chem. Soils, Ind. Farm Prod. Research Div., Misc. Pub.* 327, *ber,* 1938.)

*c. Some Special Uses.* Alcohol manufactured from corn is considere to be especially desirable for certain uses, on account of its freedom fro foreign odors and flavors. For example, such alcohol may be used in th manufacture of perfumes, flavoring extracts, and high-quality medicinals

**4. Ethyl Alcohol from Wheat.** Almost 700 million gal. of alcoho were produced from wheat and its products during the period from lat 1942 to July, 1945, according to Boruff and Van Lanen (1947). Whea was used as a carbohydrate source because of the huge demand for alcohol the shortage of molasses, and the surplus of wheat during the early year of World War II. However, the distillers were not experienced in the us of wheat and often did not have the best facilities for processing it or fo recovering the by-products. Furthermore, there was considerable foamin during fermentation when the concentration of wheat was high.

A Wheat-Alcohol Research Committee was appointed by the Wa Production Board early in World War II to help solve the problem incumbent upon the utilization of wheat. The Northern Regional Research Laboratory was selected as the coordinating agency. A summary of conferences and collaborative research was published by the laboratory i March, 1944.

Stark, Kolachov, and Willkie (1943) reported their findings on the use of wheat for alcohol production. They found that the white or soft red winter (red winter subclass) types of wheat were best suited for alcohol production, that durum and hard red spring wheats were generally not suitable because of their lower starch contents, and that hard red winter wheat was intermediate between these groups in acceptability. They observed that pressure-cooking by the batch or continuous-process methods or atmospheric mashing at 155°F. were satisfactory.

The continuous pressure-cooking of wheat was carried out as follows: Soft red winter wheat (red winter grade 1) was ground in such a manner that 55 per cent remained above a 20-mesh screen. The ground wheat was mixed with water and the premalt, precooked at 145°F. for 10 min. passed through a jet heater at 350°F. and held for 60 sec., then cooled to 152°F. (66.7°C.), in a continuous operation. In the atmospheric mashing process at 155°F., water (22 gal. per bu.) was heated to 110°F. and the wheat, ground as described above, was added. The mixture was heated to 155°F. in 45 min. and held at this temperature for 1 hr. Thereafter the mash was cooled to the conversion temperature (145 to 148°F.) and the conversion agents were added. A period of 30 min. was permitted for conversion.

The problem of foaming may usually be overcome by using mixture of corn and wheat in which the wheat represents not more than 35 to 4 per cent of the mixture.

The recovery of distillers' grain and solubles was low early in World War II, owing to lack of adequate facilities in the distilleries, but wa high by 1945. Reports concerning by-products and their recovery hav been made by Miller (1943), Jacobs (1946), Boruff (1947), and others.

**5. Ethyl Alcohol from Dehydrated Sweet Potatoes.** The productio of ethanol from dehydrated sweet potatoes of the Puerto Rico and L-4 eties has been studied by Jump, Zarow, and Stark (1944). The dehy- ed potatoes were in the form of sticks about ⅛ to ¼ in. in diameter up to 1½ in. in length. The potatoes, ground or unground, were pre- d for fermentation by mixing with stillage and/or water, by cooking, by saccharification with barley malt. The mash bill used in the fermentations was usually 89 per cent dehydrated sweet potatoes and er cent barley malt. Previous to cooking (at 35 gal. of water and age per 56-lb. bushel of potatoes), the water and stillage, in a ratio of were heated to 120°F. The potatoes, ground or unground, were then ed together with 1 per cent of the malt for premalting purposes.

tmospheric cooking at a pH of 5.3 was accomplished during the rais- of temperature of the mash to 206°F. in 1 hr., the holding of the tem- ature between 206 and 202°F. for 1.5 hr., and cooling to 145°F. in in. Pressure-cooking, when used, was accomplished in a similar man- except that the mash was held at 206 to 202°F. for 0.5 hr. instead .5 hr. and then was autoclaved for 1 hr. at 22 lb. of steam pressure. accharification was initiated at 145°F. In the first fermentations, of the malt was added and the mash was held for 10 min. at 145°F. n the remainder of the malt was added, and the mash was cooled to setting temperature. In an alternate method, all of the malt, except used for premalting, was added and the mash was maintained at F. for 60 min.

he stillage used was of two kinds: wheat-milo and sweet potato. ing the first fermentations, sufficient stillage was added after the harification process to bring the concentration of stillage in the mash 8 per cent.

concentration of 45 gal. of mash per bushel of grain was used in ng the fermentors.

ble 36 shows alcohol yields from ground and unground dehydrated t potatoes.

mp, Zarow, and Stark concluded as follows as a result of their es:

> L-4-5 variety was better than the Puerto Rico variety for alcohol produc- Grinding was not necessary before cooking, although it increased yields ly. Best yields of alcohol were obtained when the final mash contained er cent by volume of a wheat-milo stillage. Yields were distinctly lower sweet potato stillage or with no stillage. There were no significant differences ohol yields from mashes cooked by atmospheric or pressure methods. In a run, yields of 4.77 proof gallons of alcohol per bushel were obtained with o Rico sweet potatoes, and 5.44 proof gallons per bushel with the L-4-5 y. Cooking and conversion (saccharification) of dehydrated sweet potatoes e carried out successfully in grain distilleries without use of grain other than or conversion and without any change in the equipment.

**Ethyl Alcohol from Jerusalem Artichokes.** The Jerusalem arti- . or girasol (*Helianthus tuberosus*), native to North America, is a that has been studied in recent years as a possible source of se and/or industrial alcohol. Although this plant is cultivated on a

TABLE 36. ALCOHOL YIELD FROM DEHYDRATED SWEET POTATOES, GROUND AND UNGROUND *, †

| Potato | Grind | Balling | Final data | | | |
|---|---|---|---|---|---|---|
| | | | Sugar, g./ 100 ml. | Alcohol yield, proof gal./bu. | | Plant ficienc per ce |
| | | | | Wet | Dry | |
| L-4-5 (shredded) .......... | Unground | 0.6 | 0.72 | 6.06 | 6.50 | 92.5 |
| | Coarse | 0.6 | 0.80 | 6.16 | 6.61 | 94.1 |
| | Fine | 0.6 | 0.80 | 6.16 | 6.61 | 94.1 |
| Puerto Rico (shredded) ... | Unground | 1.7 | 0.78 | 5.76 | 6.27 | 86.6 |
| | Coarse | 1.9 | 0.96 | 5.70 | 6.32 | 87.3 |
| | Fine | 1.8 | 0.85 | 5.76 | 6.27 | 86.7 |
| Puerto Rico .............. | Unground | 1.2 | 0.76 | 5.76 | 6.33 | 87.3 |
| | Coarse | 1.2 | 0.76 | 5.88 | 6.47 | 89.2 |
| | Fine | 1.2 | 0.75 | 5.89 | 6.48 | 89.3 |

* J. A. Jump, A. I. Zarow, and W. H. Stark, *Ind. Eng. Chem.*, **36**: 1138 (194 Copyright 1944 by the American Chemical Society and reprinted by permission the copyright owner.

† Represents the average of three fermentors.

large scale in some parts of Europe, it is not an important crop in t United States at present, except in a few localities.

The Jerusalem artichoke is rich in the polysaccharide inulin ($C_6H_{10}O_5$ which is readily hydrolyzed to levulose.

*a. Yield per Acre.* The Jerusalem artichoke has been studied by Bo well and his associates (1936) in considerable detail. Investigating 2 varieties of the Jerusalem artichoke, all grown in three different parts the United States for 3 different years, they found that the mean yie per acre was 6.58 tons at Urbana, Ill., 16.73 tons at Corvallis, Or and 8.74 tons at Washington, D.C. The mean yield of the 20 varieti at all three places for 3 years was 10.69 tons per acre.

*b. Sugar Content.* The sugar content of the tubers, after hydrolys varied in different seasons. A 6-year mean analysis of the 20 varieties Jerusalem artichokes investigated above showed 13.33 per cent levulo and 16.38 per cent total sugars.

*c. Storage.* If an alcohol plant is to be operated continuously, a co stant supply of the raw material must be available. The fresh Jerusale artichoke tuber cannot be stored satisfactorily. However, certain metho for storing the product have been worked out. McGlumphy and h associates (1931) showed that the thinly sliced tuber could be sati factorily desiccated and stored without sugar loss.

Both the dried and the fresh tuber chips may be extracted by water diffusion batteries, but such extracts deteriorate rapidly owing to micr organisms. If the extract, which contains the soluble sugars, is evap d under reduced pressure to a total solid content of greater than 70 cent, it becomes immune to decomposition by bacteria and yeasts. storing the concentrated sirup under an atmosphere of carbon dioxide, d growth is also prevented (Underkofler et al., 1937).

he carbohydrates of the extract were stable at pH values between and 9 at temperatures as high as 110°C.

*Fermentation.* For fermentation, the sirup is diluted to yield roximately 12 per cent reducing sugars after hydrolysis, sterilized, ed, and inoculated with yeast. *S. cerevisiae*, *S. anamensis*, and espe- ly *S. pombe* have been used with satisfactory results by Underkofler his associates.

is not necessary to make a preliminary hydrolysis of the sirup or to additional nutrient substances.

*Culture of Yeast.* When the yeast was continuously cultured on ydrolyzed sirup from artichokes, its ability to produce ethanol was eased.

*Yields.* Yields of 90 per cent or more were obtained under the fore- g conditions.

**Ethyl Alcohol from Sulfite Liquor.** Sulfite liquor is produced as iste product in the manufacture of pulp from wood.

*Pulping Process.* Spruce, hemlock, or some other kind of wood is into small chips that are subsequently digested with calcium bisul- by means of heat and pressure. The sulfite liquor reaches a tempera- of 130 to 140°C. toward the end of the process. In some processes temperature may rise even higher, with the result that some of the r in the liquor is destroyed. The cellulose pulp obtained by this od is used for the manufacture of paper.

*Composition of Sulfite Liquor.* Together with each ton of pulp, is produced 8 to 10 tons of sulfite liquor, which contains from 12 per cent of total solids. One analysis (Eweson, 1936) of sulfite r showed the presence of the following products for each 1,000 kg. of ose obtained from Swedish spruce: lignin, 644 kg.; carbohydrates, g.; proteins, 15 kg.; resin and fats, 73 kg.; sulfur dioxide combined -lignin, 235 kg.; and calcium oxide combined with lignosulfonic acid, g.

e carbohydrates consisted of 49.4 per cent glucose, 15.6 per cent iose, 8.1 per cent galactose, and 26.9 per cent nonfermentable pento- (arabinose).

pproximately 65 per cent of the total reducing sugars is usually entable.

*Treatment of Sulfite Liquor.* Before the liquor is inoculated with it is necessary to remove or neutralize the sulfur dioxide, acetic and formic acid that are present. The methods commonly employed omplish this are (1) steam-stripping followed by neutralization with or calcium carbonate or (2) direct neutralization.

*Production.* The production of ethyl alcohol from sulfite waste has been described by Foth; Sankey and Rosten (1944); Joseph ); Ericsson (1947); Agar and McCarthy (1951); and others.

Ethyl alcohol is produced commercially from sulfite liquor in t United States, Canada, Germany, Sweden, and other countries.

*e. Process at Bellingham, Wash.* Ericsson (1947) described the pr duction of ethyl alcohol from sulfite waste liquor at the Bellingham plar Figure 23 shows a flow diagram for the process.

COLLECTION OF LIQUOR. Sulfite waste liquor from the digesters is di charged, together with wood pulp fibers, into blow pits that have pe forated bottoms or plates of stainless steel. The sulfite liquor is draine off and stored in a tank at a temperature of about 90°C.

CONDITIONING. From the storage tank the sulfite liquor is pumped the top of a column and flows downward over stainless-steel perforate plates, while steam introduced at the bottom of the column and flowir upward removes sulfur dioxide and other volatile materials. The sulf dioxide saved is reused. The hot sulfite liquor is pumped through screer which remove wood pulp fibers. It flows by gravity to a storage tanl Passage of the liquor through flash coolers reduces its temperature 30°C. and concentrates it by about 10 to 12 per cent. A slurry of lime added to adjust the pH to 4.5. Urea is added to the liquor as a source nitrogen.

FERMENTATION. The conditioned sulfite liquor is pumped into the firs of a series of seven interconnected fermentation tanks. Yeast from a pre vious cycle is mixed with the sulfite liquor as it flows to the fermento The medium is agitated in each tank, and the flow of liquor is on a con tinuous basis. Fermentation is carried out at 30°C.

SEPARATION OF YEAST. After the fermentation is over, in about 20 hr the fermented liquor is pumped to yeast separators. The required amoun of yeast is returned to the first fermentor for reuse.

FINAL OPERATIONS. The clarified liquor (beer) is passed by gravity t a storage tank. Then it is distilled. Finally the alcohol is rectified t 190 proof or higher, purified, and warehoused.

YIELD. The yield is 22 gal. of alcohol per ton of pulp.

CAPACITY OF PLANT. The capacity of the plant is 6,500 gal. (or more of alcohol per day.

*f. The Melle Process.* This process was developed by Les Usines d Melle[1] and involves the "reuse of yeast." The yeast from a complete alcohol fermentation is separated from the alcohol and spent liquor b means of centrifugal separators. This yeast is used again and again wit usually no appreciable loss of fermentation ability. The reuse of yeast i particularly important in the alcoholic fermentation of sulfite liquo where large quantities of yeast are essential for rapid and efficien fermentations.

**8. Ethyl Alcohol from Wood Sugars.** In order to obtain maximu yields from wood sugars, it is essential to remove inhibiting substance and to add necessary nutrients.

Several actions may be taken to remove inhibiting materials. Fo example, Leonard and Hajny (1944) found that the toxic character of t wood sugar solutions could be counteracted by the addition of reducir

---

[1] Canadian Patents 341,720; 348,549; and 402,847.

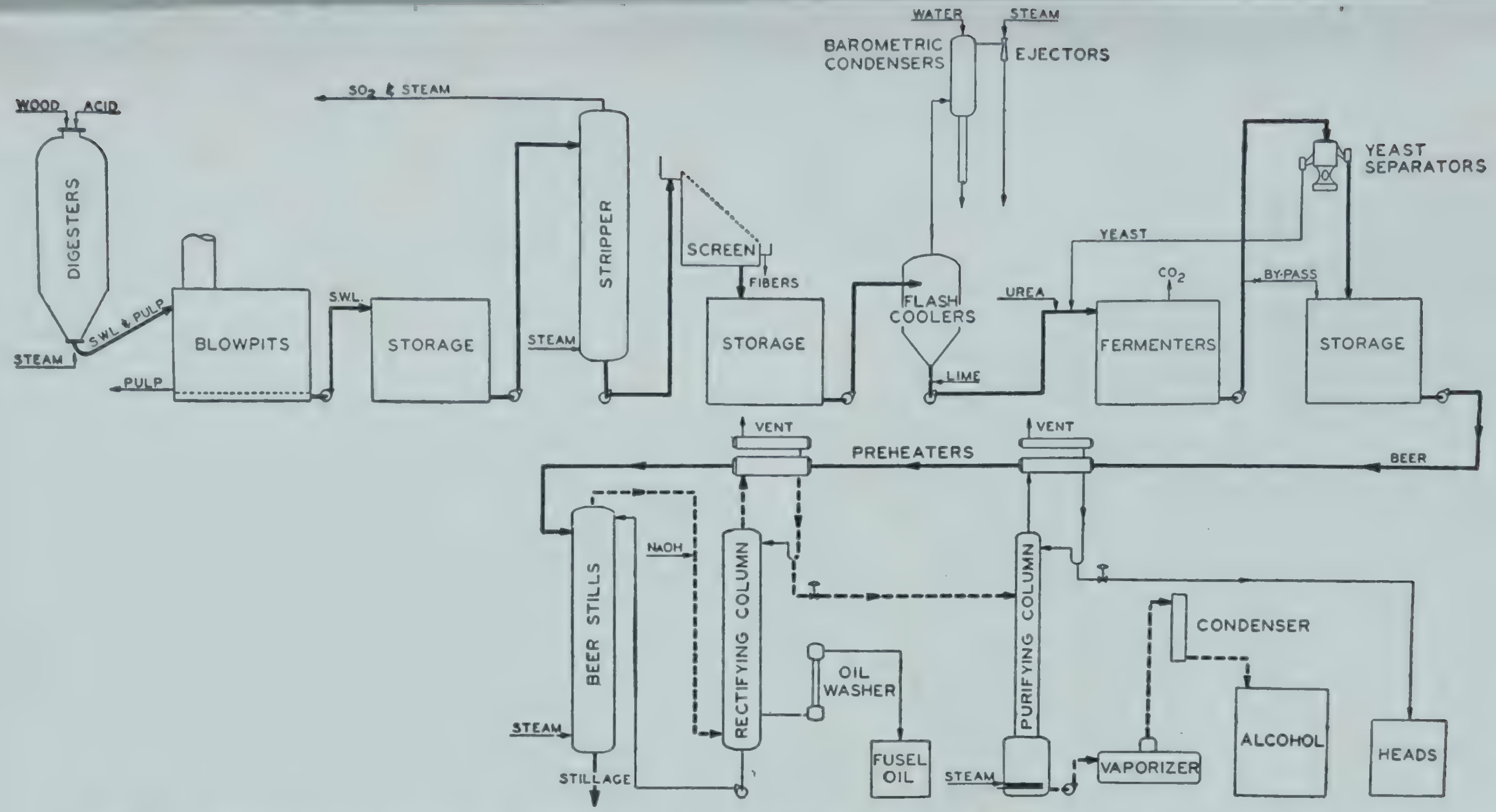

WATER
STEAM
BAROMETRIC CONDENSERS
EJECTORS
$SO_2$ & STEAM
WOOD
ACID
DIGESTERS
STEAM
SWL & PULP
BLOWPITS
PULP
S.W.L.
STORAGE
STEAM
STRIPPER
SCREEN
FIBERS
STORAGE
FLASH COOLERS
LIME
YEAST
UREA
$CO_2$
FERMENTERS
BY-PASS
YEAST SEPARATORS
STORAGE
VENT
VENT
PREHEATERS
BEER
NAOH
BEER STILLS
STEAM
STILLAGE
RECTIFYING COLUMN
OIL WASHER
FUSEL OIL
PURIFYING COLUMN
STEAM
VAPORIZER
CONDENSER
ALCOHOL
HEADS

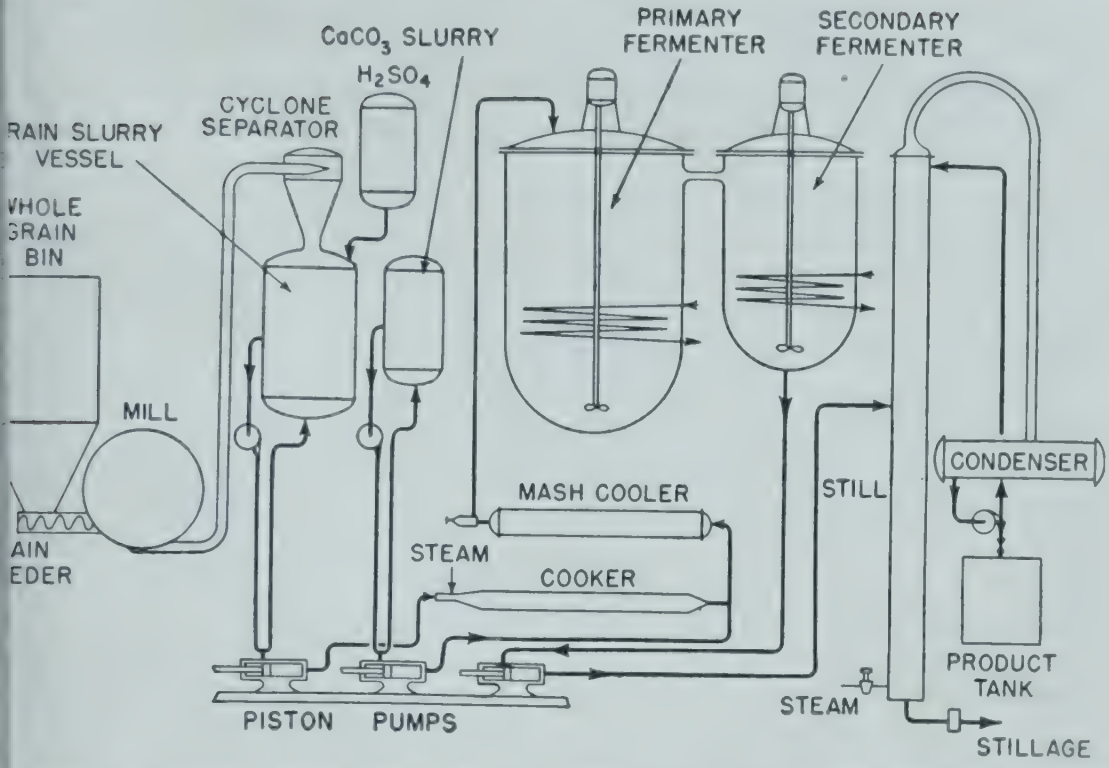
FIG. 23. Generalized flow diagram. [*Courtesy of E. O. Ericsson, Chem. Eng. Progr. (Trans. Sec.)*, 43(4): 165 (1947).]

agents, such as $Na_2SO_3$, $NaHSO_3$, $Na_2S_2O_3 \cdot 5H_2O$, $Na_2S_2O_5$, $Na_2S_2O$ $KHSO_3$, $Na_2S$, sulfite waste liquor, alkali decomposed sugar, ascorbi acid, cysteine, and reduced-iron filings. The same effect could be secure by heating neutral solutions. The amount of dissolved calcium sulfite wa decreased by neutralization at about 140°C. For good alcohol yields an avoidance of sugar losses, heating should be for 15 to 30 min. at 140°C with the solution at a pH of 4.5 to 5.2. Toxic substances were remove from the wood hydrolyzate by steam distillation, which was particularl beneficial when a quantity of greater than 0.1 per cent of furfural wa present.

Leonard and Hajny (1944) found the following method of treating an fermenting wood sugars to be successful: The acid hydrolyzate was neutralized at room temperature to a pH of 5 ± 0.2 with a slurry of calciu carbonate. The product was filtered, and 0.03 per cent by weight of sulfu dioxide as $Na_2SO_3$, $Na_2S_2O_5$, or similar reducing agent, was added. Th mixture was heated for 15 min. at 135 to 140°C. in a bomb, drawn and cooled to 30°C. The pH of the medium was adjusted to 5.8 wit sodium hydroxide. Nutrients, 0.02 per cent urea and 0.006 per cen $NaH_2PO_4$ by weight, were added, and the medium was inoculated wit 2 per cent by volume of fresh yeast (*S. cerevisiae* No. 49 of the University of Wisconsin collection) and agitated. The fermentation was usually complete in less than 20 hr. when the initial sugar concentration, as glucose, was 5 to 7 g. per 100 ml.

Harris and associates (1946) studied the fermentation of Douglas fi hydrolyzates. They observed that the fermentation time was 5 to 6 hr when the inoculum was 2 per cent by dry weight of *S. cerevisiae* (No. 49 University of Wisconsin) and 24 hr. when the inoculum was 0.5 per cent by dry weight. The yeast from an 18- to 20-hr. fermentation was used to inoculate a fresh lot of medium containing 5 per cent sugar. At the end of this fermentation the yeast was separated out and used to inoculate a new batch. This sequence was followed 59 times without loss in alcoho yield and without contamination with bacteria and other yeasts or molds It was possible to increase the rate of fermentation but not the yield o alcohol from wood sugars by the addition of 0.025 per cent Louisiana second-crop molasses. They obtained yields of 39.2 to 40 per cent based on total sugar and 47 to 47.9 per cent based on fermented sugar. In terms of a 5 per cent sugar solution, a yield of 2.64 gal. of 95 per cent alcohol per 100 gal. of hydrolyzate was obtained. From 1 ton of dry bark-free wood, 64.5 gal. of alcohol was produced.

**Two-bu.-per-day Continuous-alcohol Unit.** Altsheler and his associates (1947) have designed a continuous-process unit capable of producing 5 gal. of 190-proof alcohol in 24 hr. from 2 bu. of grain. A flow diagram of the process employed is shown in Fig. 24. The process is a follows: Corn is transported from a bin by a screw conveyor to a Raymond hammer mill where it is ground in such a manner that substantially all of it passes through a 20-mesh screen. The meal is blown by the mill to a cyclone separator, from which it falls to a slurry vessel made o Pyrex glass. It is mixed with enough water to provide a ratio of about


CaCO₃ SLURRY
H₂SO₄
PRIMARY FERMENTER
SECONDARY FERMENTER
CYCLONE SEPARATOR
RAIN SLURRY VESSEL
VHOLE GRAIN BIN
MILL
AIN EDER
MASH COOLER
STEAM
COOKER
STILL
CONDENSER
PRODUCT TANK
STEAM
STILLAGE
PISTON PUMPS

G. 24. Simplified process flow diagram. [*Courtesy of W. B. Altsheler, H. W. ollet, E. H. C. Brown, W. H. Stark, and L. A. Smith, Chem. Eng. Progr. (Trans. c.),* 43(9): 467 (1947).]

gal. per bu. Sufficient sulfuric acid is added to produce a weight con-
ntration of 1.25 per cent.

The mash is forced to a cooker tube, which is 1.5 in. in diameter and
de of copper, by a piston pump. Steam at a pressure of 150 lb. per sq.
heats it almost instantly to 340°F. It is held in the cooker for 6 min.
l then neutralized by a slurry of calcium carbonate. It is then cooled
88 to 90°F. in a heat exchanger (20 ft. of 0.25-in. copper tubing in the
m of a helix with cooling water on the outside) and discharged to the
t of two fermentors.

ermentation is continuous and is carried out in Pyrex-glass vessels,
h of which is equipped with a stirrer. The primary fermentor is
ipped with cooling coils and has a working capacity of 27 gal. It is
nected at the top with the secondary fermentor by means of an over-
v pipe. The mash remains about 8 hr. in the primary fermentor and
out 3 hr. in the secondary fermentor.

The concentration of the mash as it enters the primary fermentation
k is 1 bu. of corn in 40 gal. Yeast is added to the mash when the
cess is first started, but the rate of reproduction is sufficiently rapid
reafter to obviate the necessity of further additions. The usual con-
tration of cells is 100 million per ml. The temperature is maintained
88 to 90°F. in the primary fermentor by means of water circulating
ough coils. The pH is 4.4 to 4.6.

lcohol is stripped from the beer by 20 perforated plates located in the
er section of the still and rectified by 26 bubble-cap plates in the upper

section of the still. The alcohol vapors from the top of the column trav to a condenser, which is cooled by water.

**Yields from Various Raw Materials.** The probable average yield 99.5 per cent ethyl alcohol per ton of raw material is shown in Table 3

TABLE 37. AVERAGE YIELD * OF 99.5 PER CENT ALCOHOL PER TON †

| Material | Gal. | Material | Ga |
|---|---|---|---|
| Wheat (all varieties) | 85.0 | Yams | 27 |
| Corn | 84.0 | Potatoes | 22 |
| Buckwheat | 83.4 | Sugar beets | 22 |
| Raisins | 81.4 | Figs, fresh | 21 |
| Grain sorghum | 79.5 | Jerusalem artichokes | 20 |
| Rice, rough | 79.5 | Pineapples | 15 |
| Barley | 79.2 | Sugar cane | 15. |
| Dates, dry | 79.0 | Grapes (all varieties) | 15. |
| Rye | 78 8 | Apples | 14. |
| Prunes, dry | 72.0 | Apricots | 13. |
| Molasses, blackstrap | 70.4 | Pears | 11. |
| Sorghum cane | 70.4 | Peaches | 11. |
| Oats | 63 6 | Plums (nonprunes) | 10. |
| Figs, dry | 59.0 | Carrots | 9. |
| Sweet potatoes | 34.2 | | |

* Probable yield from a short ton of the raw material, calculated from the averag fermentable content.

† P. B. Jacobs and H. P. Newton, *U.S. Dept. Agr., Misc. Pub.* 327, December, 1938

Table 38 supplies information concerning the probable average yiel of ethyl alcohol per acre from various farm crops.

TABLE 38. AVERAGE YIELD * OF 99.5 PER CENT ALCOHOL PER ACRE †

| Material | Gal. | Material | Ga |
|---|---|---|---|
| Sugar cane (Hawaii, 18 to 22 months) | 889.0 | Pineapples | 78. |
| Sugar beets | 287.0 | Rice, rough | 65. |
| Sugar cane (Louisiana) | 268.0 | Pears | 49. |
| Jerusalem artichokes | 180.0 | Barley | 47. |
| Potatoes | 178.0 | Molasses, blackstrap | 45. |
| Sweet potatoes | 141.0 | Apricots | 41. |
| Apples | 140.0 | Oats | 36. |
| Dates, dry | 126.0 | Grain sorghum | 35. |
| Carrots | 121.0 | Buckwheat | 34. |
| Raisins | 101.7 | Wheat (all varieties) | 33. |
| Yams | 94.0 | Figs, fresh | 31. |
| Grapes (all varieties) | 90.4 | Figs, dry | 29. |
| Corn | 88.8 | Sorghum cane | 26. |
| Peaches | 84.0 | Rye | 23. |
| Prunes, dry | 82.8 | Plums (nonprunes) | 21. |

* Probable yield calculated from the average fermentable content and based o present over-all average acre yield in the United States.

† P. B. Jacobs and H. P. Newton, *U.S. Dept. Agr., Misc. Pub.* 327, December, 1938

**Government Supervision.** The purpose of Government supervision to prevent the illegal use of untaxed alcohol. Ethyl alcohol is taxed, course, to provide revenue for the Government. Should tax-free ethyl cohol be used for beverage purposes, the tax from such alcohol would lost.

Government supervision is carried out by Government officers who are ationed at all the manufacturing plants, the bonded warehouses, and ie denaturing plants, none of which can be operated without permit om the Treasury Department. The officers keep records of the raw aterials used, of the yields of ethyl alcohol, of the transfers of alcohol the bonded warehouse, of transfers of alcohol from the bonded wareuse to the denaturing plant, and of the final disposal of the alcohol. The ys to the bonded warehouse are held by the Government officers. (For rther details, consult U.S. Department of the Treasury, *Internal Reve-e Service Publication* 201.)

**Tax-paid Alcohol.** Tax-paid pure ethyl alcohol may be purchased thout permit or bond for manufacturing purposes. State regulations ust be complied with, however.

**Tax-free Alcohol.** Alcohol

> . may be withdrawn under regulations from any industrial plant or bonded rehouse tax free by the United States or any governmental agency thereof, or the several States and Territories, or any municipal subdivision thereof, or by District of Columbia, or for the use of any scientific university or college learning, any laboratory for use exclusively in scientific research, or for use any hospital or sanitarium.[1]

**Denatured Alcohol.** Denatured alcohol is alcohol to which has been ded denaturing material which destroys its character as a beverage and ders it unfit for liquid medicinal purposes. According to the U.S. partment of the Treasury, *Internal Revenue Service Publication* 368, mulas for Denatured Alcohol, Part 212 of Title 26, Code of Federal gulations, 1957, alcohol may be completely denatured or specially latured:

> ompletely denatured alcohol means denatured alcohol in which the deurants are of such a nature that such denatured alcohol may be sold and l within certain limitations without permit or bond. . . . Specially denatured hol means denatured alcohol in which the denaturant or denaturants are of a nature that such denatured alcohol may be used in a greater number of ified arts and industries than completely denatured alcohol, but, except as erwise provided in Part 182 of this chapter may be sold, possessed, and used y pursuant to permit and bond.

egulations in Part 182 relate to the production of alcohol, its removal n the industrial alcohol plant, its warehousing, its removal from the ustrial alcohol bonded warehouse, its denaturation, its removal from industrial alcohol denaturing plant, "the withdrawal, transportation, , and use of specially denatured alcohol and articles, and the withdrawal, transportation, and use of tax-free alcohol" (*Internal Reve Service Publication* 201, 1955).

---

[1] .S. Department of the Treasury, Internal Revenue Service, *Internal Revenue vice Publication* 201, 1955.

**Uses of Industrial Alcohol.** Table 39 shows some important uses industrial alcohol.

**Fusel Oil.** Fusel oil, which forms 0.1 to 0.7 per cent of the crude d tilled spirit, is a mixture of amyl and isoamyl alcohols, principally, w smaller quantities of isobutyl and normal propyl alcohols and traces acids, esters, and aldehydes. The following typical analysis is given Delbrück for the fusel oil obtained from the fermentation of potatoe amyl alcohol, 68.76 per cent; isobutyl alcohol, 24.35 per cent; *n*-prop alcohol, 6.85 per cent; and acids, esters, and furfural, 0.04 per cent. T composition and the quantity of fusel oil formed varies, however, accor ing to the raw materials used and the nature of the fermentation. F example, 1 gal. of fusel oil may be obtained for each 1,000 gal. of ethan in the fermentation of molasses, but larger quantities may be obtaine from potatoes and corn.

Fusel oil is used principally as a lacquer solvent. It is usually n refined or separated into its components.

A further discussion of some of the constituents of fusel oil will b found in Chap. 5.

## Some References concerning the Ethanol Fermentation

Agar, H. D., and J. L. McCarthy: Fermentation to Alcohol of Concentrated Calcium Magnesium, and Ammonium Sulfite Waste Liquors, *Tappi,* **34**(3): 126–13 (1951).

Anonymous: Former Waste Product Now Yields 7,500 Gallons of Alcohol Daily *DeLaval Centrifugal Rev.,* **13**(2): 3–4 (1947).

Anonymous: General Plans of Operation and List of Equipment for Whey Fermenta tion by Yeast, BDIM-1011, U.S. Department of Agriculture, Bureau of Dair Industry, May, 1947.

Atwater, W. O., and A. P. Bryant: The Chemical Composition of American Foo Materials, *U.S. Dept. Agr., Bull.* 28, rev. ed., 1906.

Barron, H.: "Distillation of Alcohol," Joseph E. Seagram & Sons, Inc., Division o Education, Louisville, Ky., 1944.

Bernhauer, K.: "Gärungschemisches Praktikum," 2d ed., Springer-Verlag, Berlin Vienna, 1939.

Bilford, H. B., R. E. Scalf, W. H. Stark, and P. J. Kolachov: Alcoholic Fermentatio of Molasses, Rapid Continuous Fermentation Process, *Ind. Eng. Chem.,* **34** 1406–1410 (1942).

Boruff, C. S., and J. M. Van Lanen: The Fermentation Industry during Worl War II, *Ind. Eng. Chem.,* **39**: 934–937 (1947).

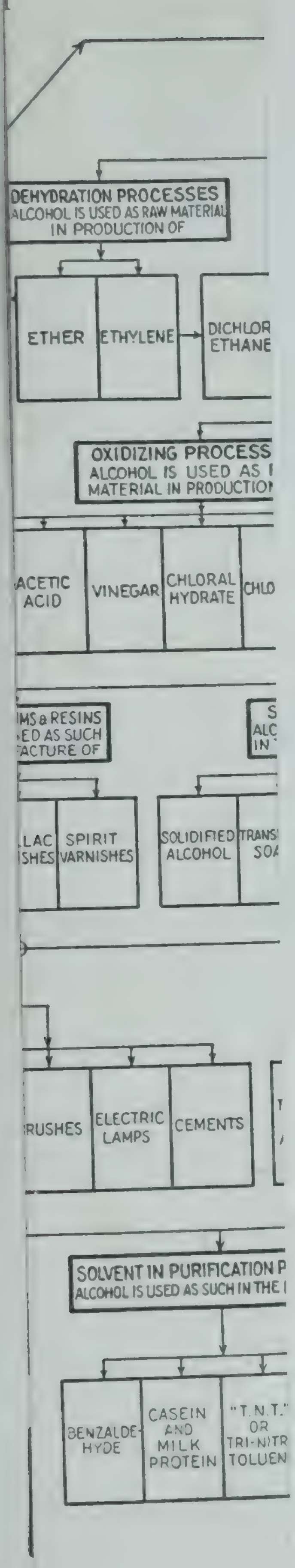
Borzani, W., and M. Falcone: Control of Starter Preparation in Fermentation o Molasses, *Jour. Agr. Food Chem.,* **1**: 1070–1071 (1953).

Boswell, V. R., C. E. Steinbauer, M. F. Babb, W. L. Burlison, W. H. Alderman, an H. A. Schoth: Studies on the Culture and Certain Varieties of the Jerusalem Artichoke, *U.S. Dept. Agr., Tech. Bull.* 514, May, 1936.

Browne, H. H.: Ethyl Alcohol from Fermentation of Lactose in Whey, *Ind. Eng Chem., News Ed.,* **19**: 1271 (1941).

Chatfield, C., and G. Adams: Proximate Composition of Fresh Vegetables. *U.S Dept. Agr., Circ.* 146, 1931.

——— and L. I. McLaughlin: Proximate Composition of Fresh Fruits, *U.S. Dep Agr., Circ.* 50, 1928.


DEHYDRATION PROCESSES
ALCOHOL IS USED AS RAW MATERIAL
IN PRODUCTION OF
ETHER
ETHYLENE
DICHLOR
ETHANE
OXIDIZING PROCESS
ALCOHOL IS USED AS
MATERIAL IN PRODUCTIO
ACETIC ACID
VINEGAR
CHLORAL HYDRATE
CHLO
IMS & RESINS
ED AS SUCH
ACTURE OF
LAC
ISHES
SPIRIT VARNISHES
S
ALC
IN
SOLIDIFIED ALCOHOL
TRANS
SOA
RUSHES
ELECTRIC LAMPS
CEMENTS
SOLVENT IN PURIFICATION P
ALCOHOL IS USED AS SUCH IN THE
BENZALDEHYDE
CASEIN AND MILK PROTEIN
"T.N.T." OR TRI-NITR TOLUEN

aassen, H.: Die Wirtschaftlichkeit der Herstellung von Holzzucker, *Chem. Ztg.*, **56**: 989 (1932).

leman, D. A. (chairman): "Methods for the Analysis of Cereals and Cereal Products. Reference Tables," American Association of Cereal Chemists, Lancaster, Pa., 1928.

nn, P. M.: Industrial Alcohol from Wood Waste, *Chem. Products*, November-December, 1945.

iesson, E. O.: Alcohol from Sulfite Waste Liquor, *Chem. Eng. Progr., Trans. Sect.*, **43**(4): 165–167 (1947).

eson, E. W.: Yeast from Wood, *Chem. Inds.*, **38**: 573 (1936).

ith, W. L., and J. A. Hall: Ethyl Alcohol from Waste Wood by a Modified Scholler Process, *Chem. Eng. News*, **22**(7): 525–526 (1944).

th, G.: "Handbuch der Spiritus Fabrikation," Paul Parey, Berlin, Chap. 19.

rdani, M.: Alcool da materiali cellulosici, *Chimica e industria (Milan)*, **21**: 265–272 (1939).

ay, W. D.: The Acclimatization of Yeast to High Concentrations of Glucose: The Subsequent Effect on Alcohol Tolerance, *Jour. Bact.*, **52**(6): 703 (1946).

rris, E. E.: Industrial Alcohol from Wood Waste, *Southern Lumberman*, Dec. 15, 1945.

——, G. J. Hajny, M. Hannan, and S. C. Rogers: Fermentation of Douglas Fir Hydrolyzate by *S. cerevisiae*, *Ind. Eng. Chem.*, **38**: 896–904 (1946); also *U.S. Dept. Agr., Forest Service, Forest Products Lab., Mimeo.* R1618, Madison, Wis., June, 1946.

obs, P. B.: Alcohol from Agricultural Commodities, *U.S. Dept. Agr., Rept. AIC*-95, revised, February, 1947.

—— and H. P. Newton: Motor Fuels from Farm Products, *U.S. Dept. Agr., Misc. Pub.* 327, December, 1938.

——, R. K. Wright, and F. M. Hildebrandt: General Purpose Fermentation Plant, *Ind. Eng. Chem.*, **40**(4): 759–764 (1948).

eph, H. G.: Industrial Alcohol Production from Sulfite Waste Liquor by the Ontario Paper Co., Ltd., *Sewage and Ind. Wastes*, **19**(1): 60–69 (1947).

p, J. A., A. I. Zarow, and W. H. Stark: *Ind. Eng. Chem.*, **36**: 1138 (1944).

achov, P., and L. W. Nicholson: Carbohydrate Sources for Ethyl Alcohol Production, *Econ. Bot.*, **5**(1): 60–81 (1951).

ssman, F. W.: The Manufacture of Ethyl Alcohol from Wood Waste, *U.S. Dept. Agr., Bull.* 983, 1922.

ard, R. H., and G. J. Hajny: Fermentation of Wood Sugars to Ethyl Alcohol, *Ind. Eng. Chem.*, **37**: 390–395 (1945).

Usines de Melle: Canadian Patents 341,720; 348,549; and 402,847.

ty, L. C., and C. W. Phelps: Gasoline-alcohol Blends in Internal-combustion Engines, *Ind. Eng. Chem.*, **20**: 222 (1938).

mann, A. J.: Alcohol in War, *Wallerstein Labs. Communs.*, **5**(15): 131–137 (1942).

Glumphy, J. H., J. W. Eichinger, R. M. Hixon, and J. H. Buchanan: Commercial Production of Levulose, *Ind. Eng. Chem.*, **23**: 1202 (1931).

ta, T. N.: "Power Alcohol for India," University of Bombay, Department of Chemical Technology, Bombay, India, 1945.

ler, E. S.: Industrial Alcohol from Wheat, *Am. Miller*, June, 1943.

n, W. L.: Continuous Fermentation, *Sugar*, **43**(2): 36 (1948).

osa, M., H. H. Browne, and E. O. Whittier: Ethyl Alcohol from Whey, *Jour. Dairy Sci.*, **30**(4): 263–269 (1947).

E. W., W. H. Stark, L. A. Smith, and E. E. Allen: Alcoholic Fermentation of Acid-hydrolyzed Grain Mashes, *Ind. Eng. Chem.*, **40**(6): 1154–1158 (1948).

nan, J. F., E. G. Locke, and G. K. Dickerman: The Production of Wood Sugar n Germany and Its Conversion to Yeast and Alcohol, *FIAT Final Report No.* 499, Nov. 14, 1945.

ey, C. A., and M. M. Rosten: Alcohol from Waste Liquor, Wartime Meeting No. of *Pulp & Paper Mag. Can.*, 1944.

Scholler, H.: Die Gewinnung von Zucker, Spiritus und Futterhefe aus Holz a Rohstoff, *Chem. Ztg.*, **60**: 293 (1936).

Sherwood, J. R.: Some Industrial Fermentations, *Australian Chem. Inst. Jour.* *Proc.*, **14**: 221–232 (June, 1947).

Skinner, W. W.: Industrial Alcohol, *U.S. Dept. Agr., Rept. AIC*-3, *NM*-235, Marc 1943.

Stark, W. H., S. L. Adams, R. E. Scalf, and P. Kolachov: Laboratory Cookin Mashing, and Fermentation Procedures, *Ind. Eng. Chem., Anal. Ed.*, **15**: 443–44 (1943).

———, P. Kolachov, and H. F. Willkie: Wheat as a Raw Material for Alcohol Production, *Ind. Eng. Chem.*, **35**: 133–137 (1943).

Thorne, C. B., R. L. Emerson, W. J. Olson, and W. H. Peterson: Evaluation o Malts for Production of Alcohol from Wheat, *Ind. Eng. Chem.*, **37**: 1142–114 (1945).

Tousley, R. D.: Alcohol Economics Will Determine Future Production Processe *Chem. Eng.*, **52**(10): 120–123 (October, 1945).

Underkofler, L. A., W. K. McPherson, and E. I. Fulmer: Alcoholic Fermentatio of Jerusalem Artichokes, *Ind. Eng. Chem.*, **29**: 1160 (1937).

U.S. Department of Agriculture, Northern Regional Research Laboratory: The Production of Ethyl Alcohol from Wheat, March, 1944.

U.S. Department of the Treasury, Bureau of Industrial Alcohol, Relative to the Production, Tax Payment, etc., of Industrial Alcohol and to the Manufacture, Sale, and Use of Denatured Alcohol, Regulations 3, 1931.

———, Internal Revenue Service, Formulae for Completely and Specially Denatured Alcohol, Appendix to Regulations 3, 1939.

Willkie, H. F., and P. J. Kolachov: "Food for Thought," Indiana Farm Bureau, Inc., Indianapolis, 1942.

## Some Patents

Bergius, F., H. Koch, and H. Zimmerman: Process for Obtaining Alcohol by Fermentation of Liquids Containing Carbohydrate, U.S. Patent 2,146,326, Feb. 7, 1939.

Christensen, L. M., and E. I. Fulmer: Production of Ethyl Alcohol from Artichoke Tubers, U.S. Patent 2,085,003, 1937.

Hildebrandt, F. M.: Alcoholic Fermentation Process and Product Thereof, U.S. Patent 1,921,991, Aug. 8, 1933.

Karsch, W.: Fermentation Process, U.S. Patent 2,155,134, Apr. 18, 1939.

Kitzmeyer, E. L.: Method of Propagation of Culture Yeast, U.S. Patent 2,150,329, Mar. 14, 1939.

Legg, D. A.: Yeast Fermentation Process, U.S. Patent 2,026,237, Dec. 31, 1935.

Magné, J. H. P.: Apparatus for Cultivating Yeast, Mucorini, Molds, or Bacilli, U.S. Patent 1,212,656, 1917.

Placěk, A.: Process for Producing Ethyl Alcohol from Wood or Other Cellulose-containing Materials, U.S. Patent 1,888,935, Nov. 22, 1932.

Scholler, H.: Purifying Wood-sugar Wort, German Patent 676967, June 16, 1939.

Schwarz, R., and A. R. Erda: Pure Culture Apparatus, U.S. Patent 2,147,271, Feb. 14, 1939.

Wagner, T. B.: Manufacture of Ethyl Alcohol from Indian Corn, U.S. Patent 2,132,250, Oct. 4, 1938.

Wickerham, L. J.: A Method for Producing Ethyl Alcohol, U.S. Patent 2,431,004, Nov. 18, 1947.

## Some References concerning Continuous Processes for Making Alcohol by Fermentation

Adams, S. L., and R. E. Hungate: Continuous Fermentation Cycle Time, *Ind. Eng* *Chem.*, **42**: 1815–1818 (1950).

Altsheler, W. B., H. W. Mollet, E. H. C. Brown, W. H. Stark, and L. A. Smith:

Design of a Two-bushel per Day Continuous Alcohol Unit, *Chem. Eng. Progr., Trans. Sect.,* **43**(9): 467–472 (1947).

ionymous: Alcohol on the Hoof, Seagram's Designs Mobile Continuous Process Alcohol Unit, *Chem. Inds.,* October, 1946.

sai, T., U. Kiyomoto, and K. Tetsuia: *Jour. Agr. Chem. Soc. Japan,* **26**: 564 (1952); **27**: 586 (1953).

orzani, W.: Fermentação Alcoólica Contínua de Mosto de Melaço, Tese de Concurso à Docência Livre da Cadeira N.° 29, "Bio-Quimica," da Escola Politécnica da Universidade de São Paulo, São Paulo, Brazil, 1952.

——: Brazil Patent 44,531, Oct. 27, 1953.

——: Contribuição ao Estudo da Fermentação Alcoólica Contínua de Mosto de Melaço, Tese apresentada à Comissão Julgadora do Concurso à Cátedra de Bio-Quimica da Escola Politécnica da Universidade de São Paulo, São Paulo, Brazil, 1955.

——: Alcoholic Fermentation of Blackstrap Molasses Continuous Fermentation, *Jour. Agr. Food Chem.,* Vol. 5, 1957.

Becze, G., and M. Rosenblatt: Continuous Fermentation, *Am. Brewer,* **76**(2): 11 (1943).

nalburg, A. M.: Russian Patents 64,934, July 31, 1945*a*, and 65,004, Aug. 31, 1945*b*.

eussler, O.: German Patent 744682, Nov. 25, 1953.

iffner and Kuffner: Austrian Patent 134,100, July 10, 1933.

alchenko, A. L., and M. P. Chistiachov: Russian Patent 77,813, Dec. 31, 1949.

ariller, C., J. Mejane, M. Martraise, and S. Tourliere: *Inds. agr. et aliment. (Paris),* **69**: 775 (1952).

attos, A. R.: U.S. Patent 2,451,156, Oct. 12, 1948.

ven, L. W.: *Sugar,* **43**(2): 36 (1948).

## CHAPTER 5

# FACTS AND THEORIES CONCERNING THE MECHANISM OF ETHYL ALCOHOL FERMENTATION

**Some Early Researches.** In the earliest studies involving the mechanism of the ethyl alcohol fermentation, one was concerned only with the initial and end products. Black (Frey, 1930) in the eighteenth century, stated that ethyl alcohol and carbon dioxide were the only products formed from sugar during the ethanol fermentation. Lavoisier, in 1789, carried out quantitative studies on this fermentation and found, besides ethyl alcohol and carbon dioxide, another product to which he gave the name "acetic acid." From 95.9 parts of sugar he obtained 57.7 per cent ethyl alcohol, 33.3 per cent $CO_2$, and 2.5 per cent acetic acid. Lavoisier's is believed to be the first quantitative work concerning the ethanol fermentation, although his results were incorrect.

Gay-Lussac investigated more intensively the field of research opened up by Lavoisier. He formulated the following equation to show what he believed was the essential chemical reaction of the ethanol fermentation:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} = \underset{\text{Ethyl alcohol}}{2C_2H_5OH} + \underset{\text{Carbon dioxide}}{2CO_2}$$

From 45 parts of glucose, he obtained 23 parts of alcohol and 22 parts of carbon dioxide.

By 1857, Pasteur had begun to publish the results of his researches on fermentation. He showed that 100 parts of sucrose gave rise to 105.4 parts of invert sugar, which in turn yielded 51.1 parts of ethyl alcohol, 49.4 parts of carbon dioxide, 3.2 parts of glycerol, 0.7 part of succinic acid, and 1 part of other substances. He thus proved that the weight relationships set forth by Gay-Lussac were in error.

Buchner, in 1897, prepared a cell-free juice from yeast (by grinding the cells with sand and subjecting them to high pressure) which possessed the ability to produce ethyl alcohol and carbon dioxide from sugar. This discovery lent great impetus to subsequent studies concerning the intermediate reactions in the formation of ethyl alcohol.

**Hexose Phosphates.** Harden and Young (1905) discovered the importance of phosphates in cell-free fermentation. They have demonstrated that inorganic phosphates disappear during the first part of the fermentation, while organic phosphates, i.e., esters of hexose, are produced. They have described hexosediphosphate and proposed the following fundamental equation for fermentation by yeast extract:

$$2C_6H_{12}O_6 + 2PO_4HR_2 = 2C_2H_5OH + 2CO_2 + C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O$$

Hexosediphosphate is an important intermediate in the schemes of Embden, Meyerhof, and others for the breakdown of carbohydrates. This compound contains 2 phosphate, or phosphoric acid, molecules attached, 1 each, to the first and sixth carbon atoms of the hexose, which appears to be fructose. The same hexosediphosphate is obtained from glucose, fructose, or mannose.

At least two hexosemonophosphates, which have different properties, have been prepared: the Robison ester (glucopyranose-6-phosphoric ester), and the Neuberg ester (fructofuranose-6-phosphoric ester). When these esters are hydrolyzed, they yield glucose and fructose, respectively.

Hexosemonophosphates have been synthesized by Levene and Raymond, Smythe, and others. A trehalosemonophosphate has been isolated by Robison and Morgan from a fermentation brought about by dried yeast.

Hexose phosphates are fermented at different rates. The diphosphate is fermented more slowly than glucose, while hexosemonophosphate is usually fermented more rapidly than the diphosphate, at a rate comparable with glucose in the initial stage only (Michaelis, 1935).

The addition of hexose phosphates to cell-free yeast extracts containng glucose removes or greatly shortens the period of induction (the pause before fermentation starts). Without phosphates, no fermentation takes place.

The addition of phosphates to a medium does not affect the rate of fermentation by living yeasts.

For further details in respect to this very important subject, the reader s referred to Harden's monograph, "Alcoholic Fermentation," and to the ibliography that follows this chapter.

**Methods of Studying the Mechanism of Fermentation.** Information oncerning the mechanism of the ethyl alcohol fermentation may be ained by studying the related mechanism of lactic acid formation by muscle extracts; by the use of cell-free yeast juice or extracts, fixation methods, selective poisons, and dialysis; and by other means.

1. *Related Mechanism of Lactic Acid Formation by Muscle Extracts.* ny productive advance in the study of the mechanism of lactic acid ormation by muscle extract has usually aided materially in the study of ne mechanism of the ethyl alcohol fermentation, and vice versa. The esearches of Embden on muscle extract exemplify the impetus that esearch on the former may do for the latter. The outstanding work of Meyerhof and his school add further proof to the valuable insight gained hrough correlative studies.

2. *Cell-free Extracts.* Since the important reactions that take place the conversion of sugar to ethyl alcohol occur normally within the ving cell, since added hexose phosphates are fermented extremely slowly not at all, and since the isolation of intermediates from the living cell

is impossible in many cases, but comparatively little information concern ing the intermediary reactions may be derived from studying such cell Accordingly, cell-free extracts are used.

Two common methods of preparing the extract are the Buchner metho (already mentioned) and the Lebedev (1911) method. In the latte method the yeast is washed thoroughly with water, dried at 25 to 30°C rubbed through a sieve, dried further, and stored until such time as a extract may be required. The extract is prepared by mixing 1 part b weight of dried yeast with 3 parts of water, incubating at 37°C. for 3 hr and filtering the extract into a vessel cooled by ice (Stephenson, 1939) It is important to use an extract that is free from living cells.

3. *Fixation Method.* The fixation method was used with success b Neuberg. In this method a sulfite, such as calcium sulfite, or dimedo (dimethyl cyclohexane-dione), is used to fix the acetaldehyde as it i formed. The fixation product is not fermented by yeast and therefor accumulates in the medium. Since acetaldehyde is a hydrogen acceptor the removal of this substance from the fermentation medium causes othe hydrogen acceptors to become active. Use is made of this fact in th production of glycerol by fermentation, where the role of sulfites is ver important.

Although pyruvic aldehyde forms an addition product with sulfites, thi product is fermentable.

4. *Selective Poisons.* Certain reactions in the normal chain of enzym reactions do not take place in the presence of selective poisons, such a monoiodoacetic acid, sodium fluoride, and other substances. The poison affect specific enzyme systems.

When monoiodoacetic acid is added to a cell-free extract containin hexosediphosphate, a mixture of triosephosphates forms as usual, which contains dihydroxyacetone phosphate and glyceraldehyde phosphate, but the reaction, in which triosephosphate is oxidized to phosphoglyceric acid with the simultaneous reduction of acetaldehyde to ethyl alcohol, does not take place, owing to a poisoning of the enzyme system. Monoiodoacetic acid does not, however, retard the decomposition of phosphoglyceric acid.

Sodium fluoride, on the other hand, prevents phosphoglyceric acid from being broken down to pyruvic and phosphoric acids, but it does not prevent triosephosphate from being converted to phosphoglyceric and glycerophosphoric acids. Accordingly, phosphoglyceric acid accumulates in the medium.

5. *Dialysis.* By means of dialysis, important constituents of yeast extract may be separated and studied.

The adenylic acid system and magnesium may be removed from yeast extract by dialysis. It is thus possible to study the reactions in which a gain or loss of phosphates is concerned.

Proteins, separated from coenzyme systems by dialysis, may be fractionated in the ways common to biochemistry.

6. *Other Means.* There are other ways in which the intermediary reactions of fermentation may be studied.

A very valuable method for studying the intermediate metabolism nvolves the use of labeled atoms.

Kluyver, Van der Lek, and others have demonstrated the value of tudying the quantitative relationships of the intermediate and end prodcts in formulating a scheme for the mechanism of fermentation.

Lohmann has obtained helpful information by determining the time equired for normal hydrochloric acid to split off the phosphates from hosphorylated compounds.

Important information has been secured by studying the fermentaility and fermentation rates of substances assumed to be intermediates.

A study of the formation and utilization of hexosemonophosphate has een made possible through the use of selected dyes. Naphthosulfonate ndophenol, brilliant alizarin blue, or rosindulin GG inhibit the fermentaon of glucose or of hexosemonophosphate but not of hexosediphosphate y yeast extract under aerobic conditions (Michaelis and Smythe, 1936). hen hexosediphosphate is added to yeast extract containing glucose and ne of these dyes, the hexosediphosphate is fermented. Contrary to expections, the quantity of inorganic phosphate does not increase with the rmentation of hexosediphosphate, but it actually decreases, for an ganic phosphate is synthesized. The reaction may be expressed as llows:

$$_6H_{10}O_4(PO_4M_2)_2 + 2M_2HPO_4 + 4ROH \rightarrow 4RPO_4M_2 + 2CH_3CH_2OH + 2CO_2 + 2H_2O\,*$$

The organic phosphate ($RPO_4M_2$) formed in the preceding equation pears to be hexosemonophosphate, a mixture of glucose, fructose, and annose monophosphates (Smythe, 1937).

**Precautions.** It must be borne in mind that the addition of subnces to a medium, or yeast extract, may, and frequently does, alter the mal reactions. Furthermore the isolation of a substance that does appear as an end product does not necessarily indicate that this stance is an intermediate in the main fermentation. Rigorous proofs, ablished by careful research, are necessary.

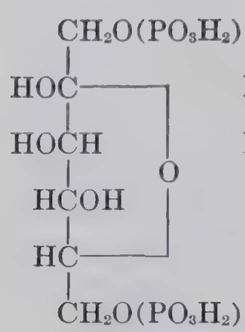
**Neuberg's Scheme.** Neuberg's scheme for the mechanism of the alco- fermentation, based partly on the fact that pyruvic acid is an interliate and is fermented at a rate equal to that of glucose and on the t that a triose can be isolated, is as follows: 1 molecule of a hexose is ken down to 2 molecules of a triose ($C_3H_6O_3$), probably a hydrated m of methylglyoxal. The triose, through loss of water, forms methyloxal or an active form of this compound. Methylglyoxal, a ketonic ehyde, is converted to pyruvic acid by oxidation, while another interliate product is reduced. Pyruvic acid is decomposed to acetaldehyde carbon dioxide under the influence of the enzyme, carboxylase. taldehyde, in an acid medium, is reduced to ethyl alcohol, while hylglyoxal is being oxidized to pyruvic acid (a simultaneous oxidation-

---

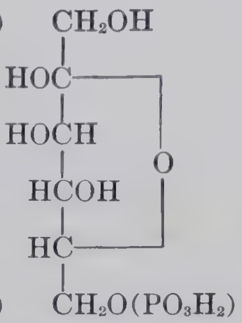
. Michaelis, V. Moraques-Gonzalez, and C. V. Smythe, *Enzymologia,* 3: 242 7).

### TABLE 40. SOME IMPORTANT INTERMEDIATE AND END PRODUCTS OF THE ETHYL ALCOHOL FERMENTATION

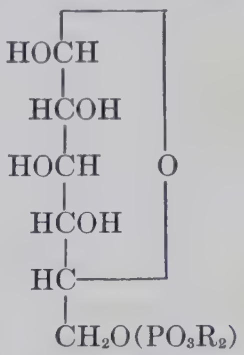
## I. *Intermediate Products*



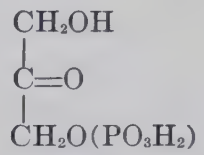
Fructofuranose 1:6-diphosphate



Neuberg ester

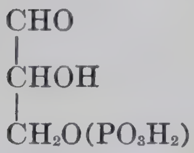


Robison ester



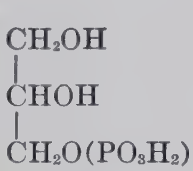
$$\begin{array}{l} CH_2OH \\ | \\ C{=}O \\ | \\ CH_2O(PO_3H_2) \end{array}$$

Dihydroxyacetone phosphate



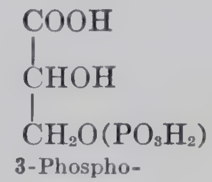
$$\begin{array}{l} CHO \\ | \\ CHOH \\ | \\ CH_2O(PO_3H_2) \end{array}$$

3-Glyceraldehyde phosphate



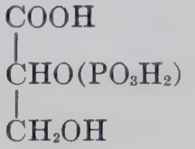
$$\begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2O(PO_3H_2) \end{array}$$

α-Glycerol phosphate



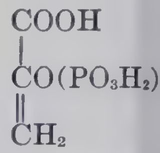
$$\begin{array}{l} COOH \\ | \\ CHOH \\ | \\ CH_2O(PO_3H_2) \end{array}$$

3-Phosphoglyceric acid



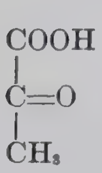
$$\begin{array}{l} COOH \\ | \\ CHO(PO_3H_2) \\ | \\ CH_2OH \end{array}$$

2-Phosphoglyceric acid



$$\begin{array}{l} COOH \\ | \\ CO(PO_3H_2) \\ \Vert \\ CH_2 \end{array}$$

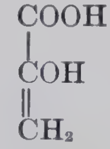
Phosphopyruvic acid



$$\begin{array}{l} COOH \\ | \\ C{=}O \\ | \\ CH_3 \end{array}$$

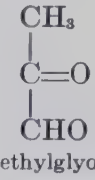
Pyruvic acid

or



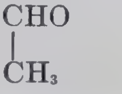
$$\begin{array}{l} COOH \\ | \\ COH \\ \Vert \\ CH_2 \end{array}$$

Pyruvic acid (enol form)



$$\begin{array}{l} CH_3 \\ | \\ C{=}O \\ | \\ CHO \end{array}$$

Methylglyoxal

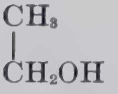


$$\begin{array}{l} CHO \\ | \\ CH_3 \end{array}$$

Acetaldehyde

## II. *End Products*

### *A*. Derived from Sugar

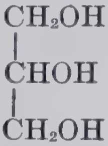


$$\begin{array}{l} CH_3 \\ | \\ CH_2OH \end{array}$$

Ethyl alcohol

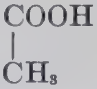
$CO_2$

Carbon dioxide



$$\begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{array}$$

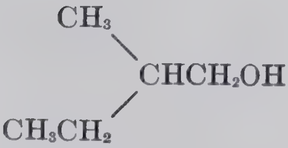
Glycerol



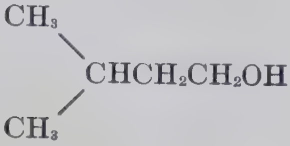
$$\begin{array}{l} COOH \\ | \\ CH_3 \end{array}$$

Acetic acid

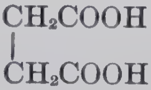
### *B*. Not Derived from Sugar



Amyl alcohol



Isoamyl alcohol

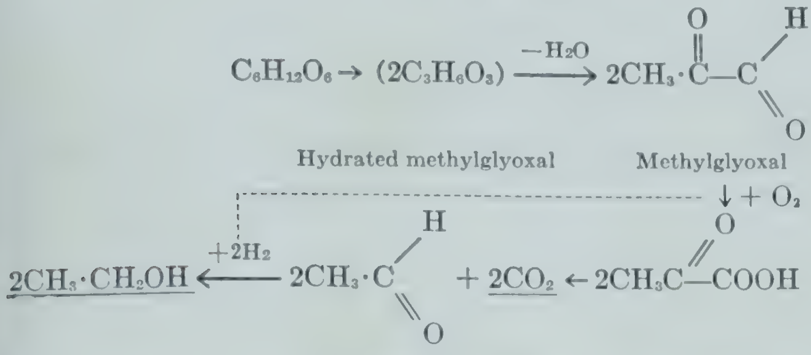


$$\begin{array}{l} CH_2COOH \\ | \\ CH_2COOH \end{array}$$

Succinic acid

eduction). The scheme thus outlined may be illustrated as shown below.

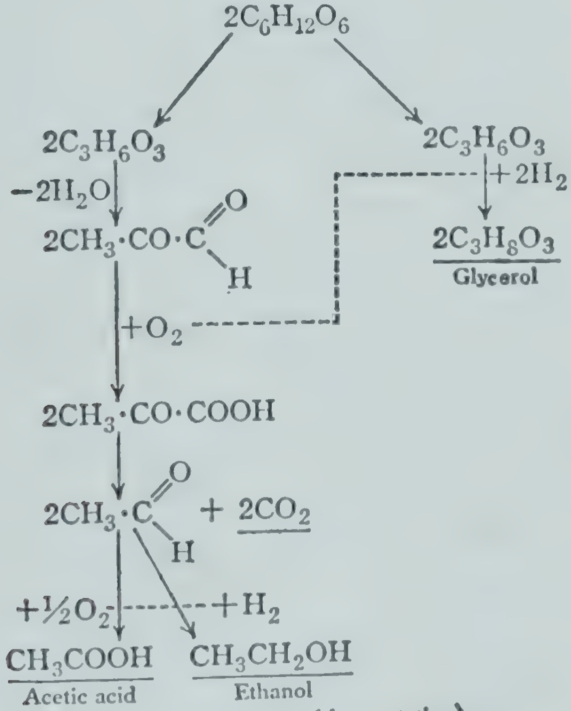
SCHEME I


$C_6H_{12}O_6 \rightarrow (2C_3H_6O_3) \xrightarrow{-H_2O} 2CH_3\cdot C(=O)-CHO$
Hydrated methylglyoxal
Methylglyoxal
$+ O_2$
$+2H_2$
$2CH_3\cdot CH_2OH \leftarrow 2CH_3\cdot CHO + 2CO_2 \leftarrow 2CH_3C(=O)-COOH$

(Neuberg's "first form" of fermentation)

In case the reaction is alkaline, a compound, believed to be unstable nd of the formula $C_3H_6O_3$, acts as a hydrogen acceptor together with cetaldehyde. The unstable $C_3H_6O_3$ compound is reduced to glycerol, hile acetaldehyde is oxidized in part to acetic acid and reduced in part o ethanol (a Cannizzaro reaction).

SCHEME II

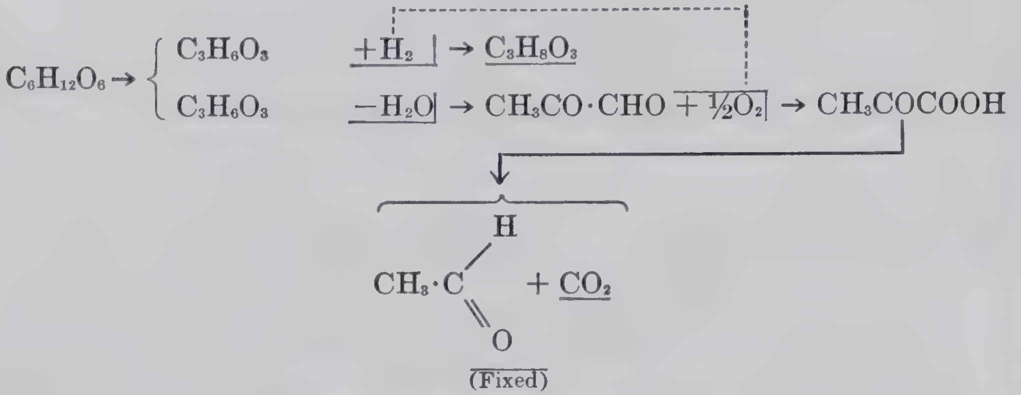

$2C_6H_{12}O_6$
$2C_3H_6O_3$
$-2H_2O$
$2CH_3\cdot CO\cdot CHO$
$2C_3H_6O_3$
$+2H_2$
$2C_3H_8O_3$
Glycerol
$+O_2$
$2CH_3\cdot CO\cdot COOH$
$2CH_3\cdot CHO + 2CO_2$
$+\frac{1}{2}O_2$
$+H_2$
$CH_3COOH$
Acetic acid
$CH_3CH_2OH$
Ethanol

(Neuberg's "third form" of fermentation)

One method of producing glycerol is by the sulfite fixation process. cetaldehyde may be fixed by sulfite before it has become reduced or idized. The aldehyde thus fixed may be recovered from the fermenta- on medium and estimated. It is evident that any acetaldehyde that s become fixed is no longer available as a hydrogen acceptor. Acetalde- yde may, then, be replaced in part by $C_3H_6O_3$ as a hydrogen acceptor.

$C_3H_6O_3$ is reduced to glycerol in proportion to the amount of acetaldehyde fixed (1 molecule of glycerol for each molecule of acetaldehyde fixed).

### SCHEME III

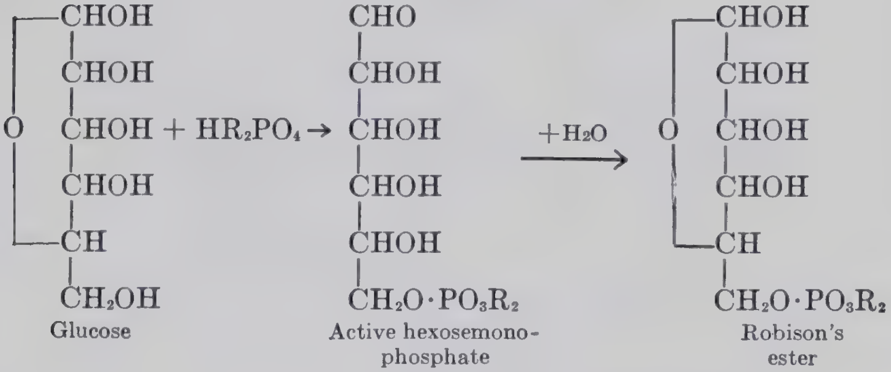

$C_6H_{12}O_6$
$C_3H_6O_3$
$C_3H_6O_3$
$+H_2$
$C_3H_8O_3$
$-H_2O$
$CH_3CO \cdot CHO$
$+ \frac{1}{2}O_2$
$CH_3COCOOH$
$CH_3 \cdot C$
H
O
$+ CO_2$
(Fixed)

(Neuberg's "second form" of fermentation)

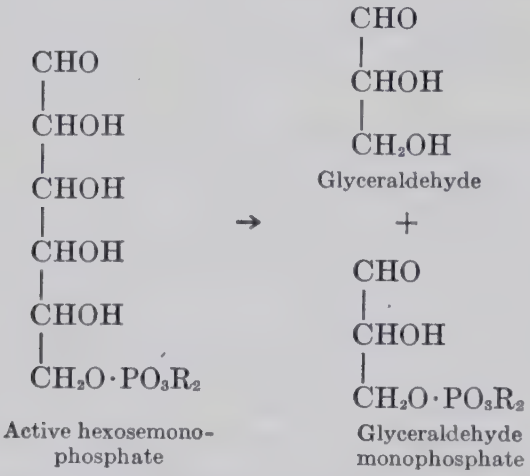
The normal fermentation, according to Neuberg, proceeds chiefly as shown in the first scheme, but some glycerol and acetic acid occur and hence the second scheme is realized in part.

### KLUYVER'S SCHEME FOR THE ALCOHOLIC FERMENTATION OF GLUCOSE *

#### I. *Initial Phosphorylation*


CHOH
CHOH
CHOH
CHOH
CH
$CH_2OH$
O
$+ HR_2PO_4$
CHO
$CH_2O \cdot PO_3R_2$
$+H_2O$
Glucose
Active hexosemono-phosphate
Robison's ester

#### II. *Oxidoreduction of Hexosemonophosphate*

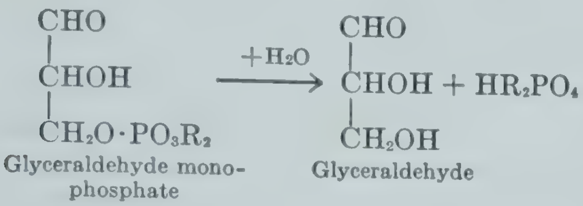

CHO
CHOH
CHOH
CHOH
CHOH
$CH_2O \cdot PO_3R_2$
$CH_2OH$
+
Active hexosemono-phosphate
Glyceraldehyde
Glyceraldehyde monophosphate

---

* A. J. Kluyver, Die bakteriellen Zuckervergärungen, *Ergeb. Enzymforsch.*, **4**: 230–273 (1935).

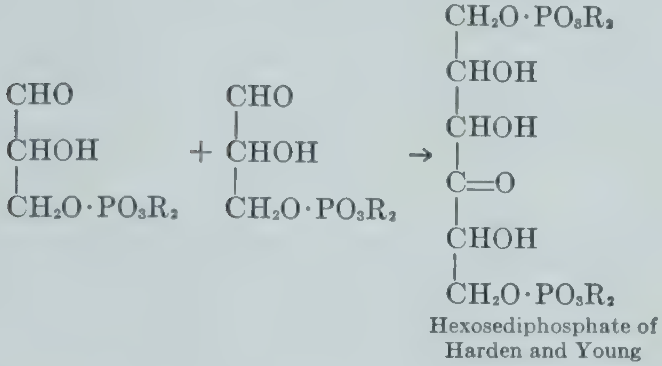
## LUYVER'S SCHEME FOR THE ALCOHOLIC FERMENTATION OF GLUCOSE * (*Continued*)

### III. *Hydrolysis of Triosephosphate*


Glyceraldehyde mono-phosphate
Glyceraldehyde

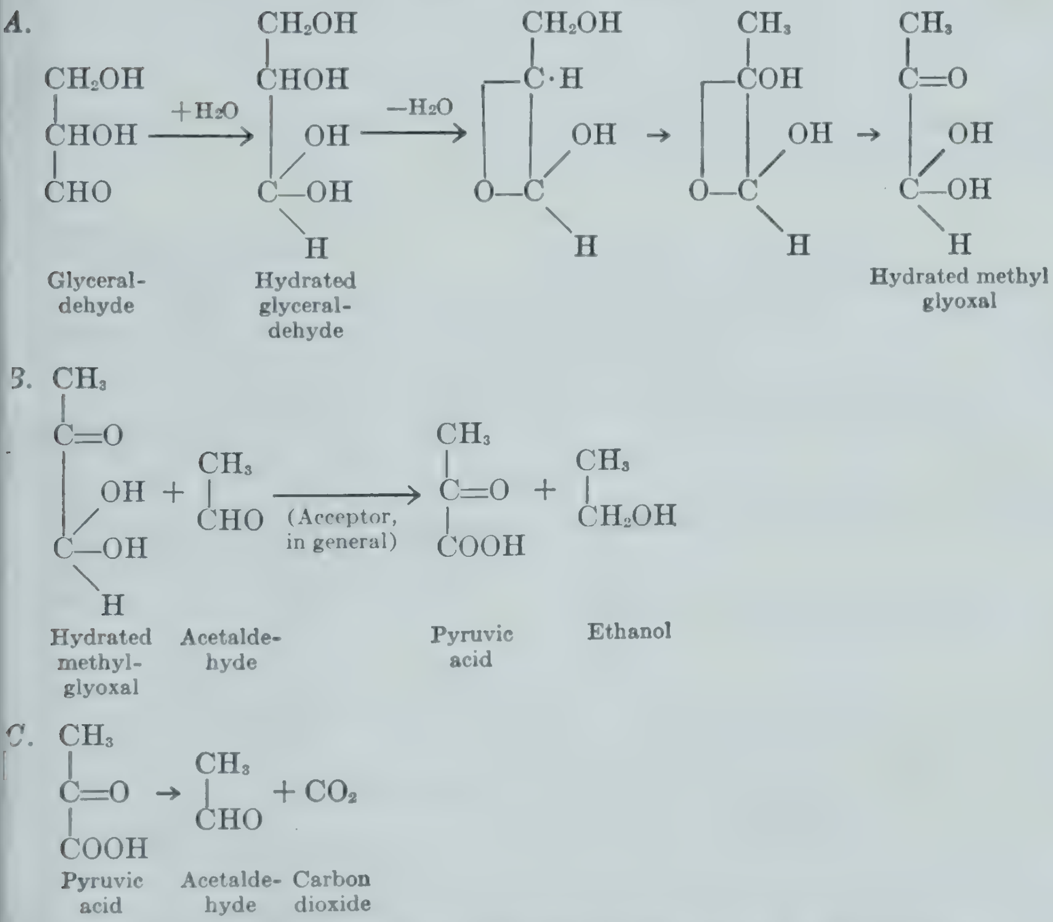
$$\mathrm{CHO{-}CHOH{-}CH_2O{\cdot}PO_3R_2} \xrightarrow{+\mathrm{H_2O}} \mathrm{CHO{-}CHOH{-}CH_2OH} + \mathrm{HR_2PO_4}$$

### Condensation Reaction


Hexosediphosphate of Harden and Young

$$\mathrm{CHO{-}CHOH{-}CH_2O{\cdot}PO_3R_2} + \mathrm{CHO{-}CHOH{-}CH_2O{\cdot}PO_3R_2} \rightarrow \mathrm{CH_2O{\cdot}PO_3R_2{-}CHOH{-}CHOH{-}C{=}O{-}CHOH{-}CH_2O{\cdot}PO_3R_2}$$

### IV. *Final Oxidoreductions*


A.
+H₂O
−H₂O
Glyceral-dehyde
Hydrated glyceral-dehyde
Hydrated methyl glyoxal
B.
(Acceptor, in general)
Hydrated methyl-glyoxal
Acetalde-hyde
Pyruvic acid
Ethanol
C.
Pyruvic acid
Acetalde-hyde
Carbon dioxide

---

A. J. Kluyver, Die bakteriellen Zuckervergärungen, *Ergeb. Enzymforsch.*, **4**: 230–
(1935).

Methylglyoxal has been isolated from the fermentation of hexo phosphate by yeast extracts in water and toluol. It has likewise isolated as a "semicarbazide" in the fermentation of sucrose by ye It is not fermentable but may be regarded as the stable isomer fermentable form.

An active hexosemonophosphate is first formed by the combinatio glucose with a phosphate. Two 3-carbon compounds, glyceraldehyde glyceraldehyde monophosphate, are the main end products for through the splitting of the active hexosemonophosphate, but Robis ester may be produced from this compound as a side product.

Glyceraldehyde monophosphate is hydrolyzed principally to gly aldehyde and phosphate, while 2 molecules may condense by a side r tion to form the hexosediphosphate of Harden and Young. The phosp liberated in the hydrolysis of glyceraldehyde monophosphate is a able to react with more glucose to form more active hexosemonop phate.

Glyceraldehyde is converted to hydrated methylglyoxal throug series of hydrogen transfer reactions. Methylglyoxal hydrate don hydrogen to a hydrogen acceptor and is changed to pyruvic acid. A aldehyde acts as the hydrogen acceptor after it is once formed, be reduced to ethanol, while pyruvic acid is being produced. Pyruvic a is decarboxylated to acetaldehyde and carbon dioxide. The acetaldeh reacts with more methylglyoxal hydrate, and the production of pyru acid, ethanol, acetaldehyde, and carbon dioxide continues.

Kluyver[1] believes that the catalytic agents responsible for the imp tant changes in his scheme for the fermentation of glucose are an oxi reductase and a phosphatase.

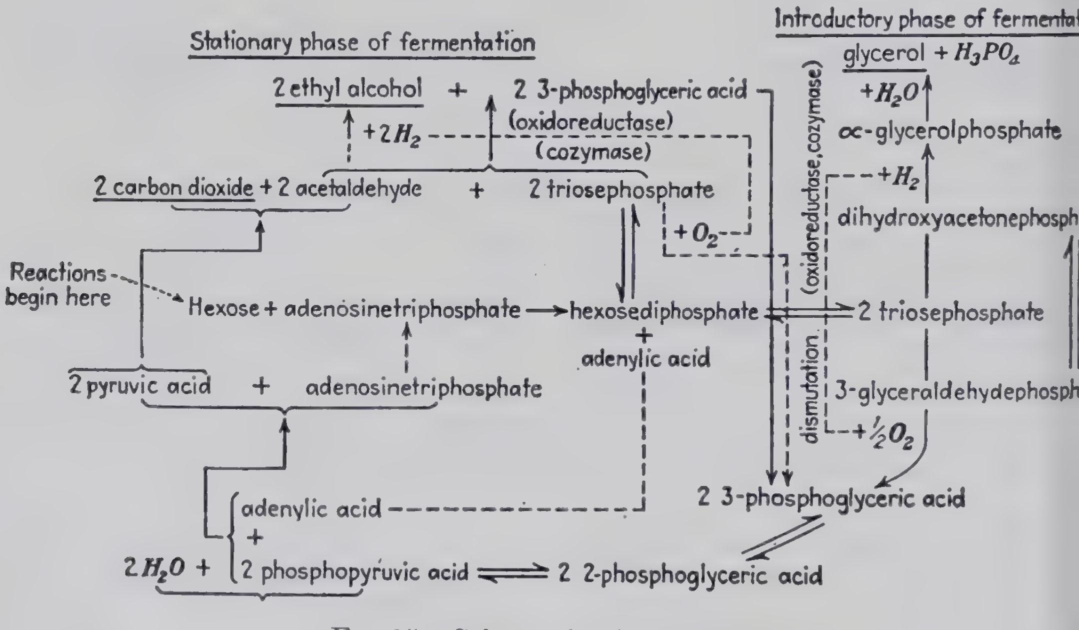

Stationary phase of fermentation
Introductory phase of fermenta
2 ethyl alcohol + 2 3-phosphoglyceric acid
+2$H_2$
(oxidoreductase)
(cozymase)
glycerol + $H_3PO_4$
+$H_2O$
∝-glycerolphosphate
+$H_2$
(oxidoreductase, cozymase)
2 carbon dioxide + 2 acetaldehyde + 2 triosephosphate
+$O_2$
dihydroxyacetonephosph
Reactions begin here
Hexose + adenosinetriphosphate → hexosediphosphate + adenylic acid
2 triosephosphate
dismutation
3-glyceraldehydephosph
+½$O_2$
2 pyruvic acid + adenosinetriphosphate
adenylic acid
+
2$H_2O$ + 2 phosphopyruvic acid ⇌ 2 2-phosphoglyceric acid
2 3-phosphoglyceric acid

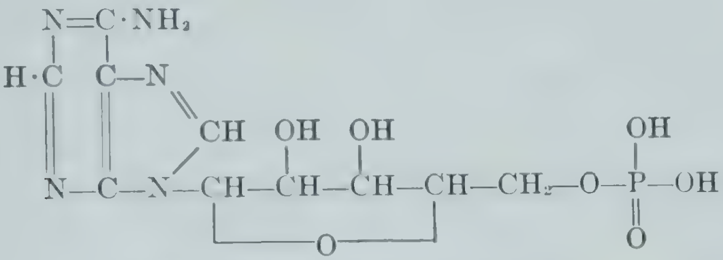
FIG. 25. Scheme for fermentation.

---

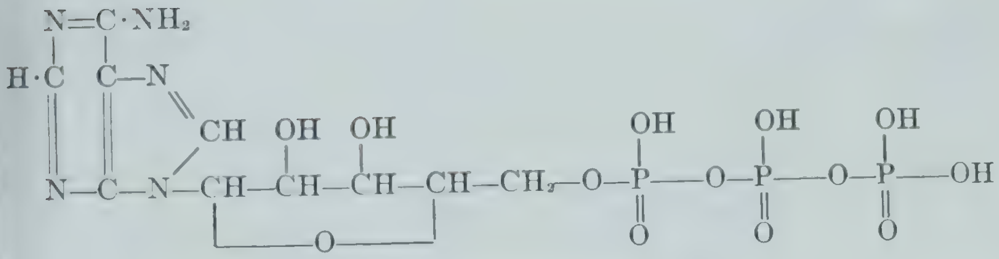
[1] For a further discussion of this subject, the reader is referred to A. J. Kluy "The Chemical Activity of Microorganisms," University of London Press, L London, 1931.

**Scheme Based on the Researches of Meyerhof and Others.** In . 25 a scheme for the intermediate reactions occurring in the ethyl ohol fermentation is shown.

The first step, in the series of reactions, is a phosphorylation of the gar (glucose, fructose, or mannose) in which a phosphorylating coen-ne, known as the "adenylic system," and obtainable in a yeast dialy-e, functions (Meyerhof, 1938). This system, or coenzyme, consists of enylic acid (adenosinemonophosphate), adenosinediphosphate, and enosinetriphosphate. The first two of these compounds have the ability pick up phosphate, while the third, adenosinetriphosphate, donates osphate. The formulas for two important members of the adenylic stem follow:



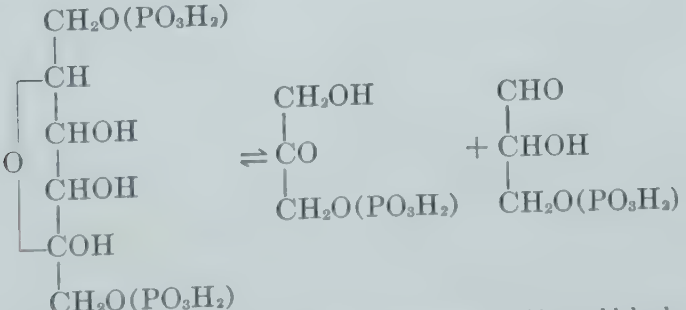
Adenylic acid, or Adenosinemonophosphate



Adenosinetriphosphate

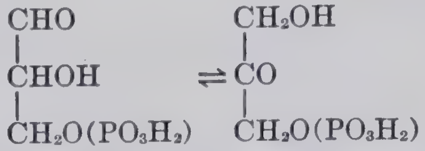
he hexose accepts phosphate from adenosinetriphosphate, the hexose-iophosphate appearing first, then the hexosediphosphate upon further sphorylation. Once the fermentation has reached the pyruvic acid e, the phosphate given off by phosphopyruvic acid becomes avail- for phosphorylating additional hexose.

ructose diphosphate, or diphosphoric acid (the common diphosphate ained from glucose, fructose, or mannose), splits to form 2 molecules riosephosphate with which it is in equilibrium:



Fructose diphosphate Dihydroxyacetone phosphate 3-Glyceraldehyde phosphate

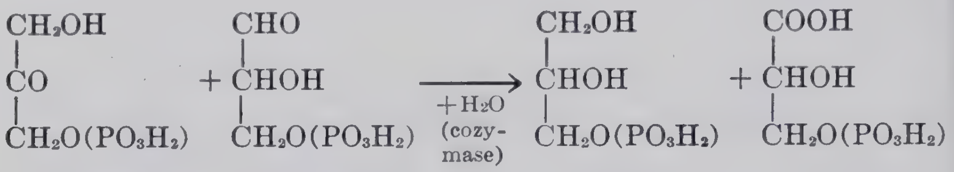
3-Glyceraldehyde is in equilibrium with dihydroxyacetone and is verted largely to the latter, according to Meyerhof.



$$\begin{array}{l}\mathrm{CHO}\\ |\\ \mathrm{CHOH}\\ |\\ \mathrm{CH_2O(PO_3H_2)}\end{array} \rightleftharpoons \begin{array}{l}\mathrm{CH_2OH}\\ |\\ \mathrm{CO}\\ |\\ \mathrm{CH_2O(PO_3H_2)}\end{array}$$

3-Glyceraldehyde phosphate Dihydroxyacetone phosphate

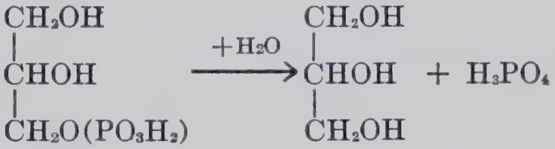
During the initial stages of fermentation (the induction period), be any acetaldehyde is produced, 2 molecules of triosephosphate dismu to form 1 molecule of 3-phosphoglyceric acid and 1 molecule of al glycerophosphate, this being an oxidation-reduction reaction in w cozymase (coenzyme I) is active:



$$\begin{array}{l}\mathrm{CH_2OH}\\ |\\ \mathrm{CO}\\ |\\ \mathrm{CH_2O(PO_3H_2)}\end{array} + \begin{array}{l}\mathrm{CHO}\\ |\\ \mathrm{CHOH}\\ |\\ \mathrm{CH_2O(PO_3H_2)}\end{array} \xrightarrow[\text{(cozymase)}]{+\mathrm{H_2O}} \begin{array}{l}\mathrm{CH_2OH}\\ |\\ \mathrm{CHOH}\\ |\\ \mathrm{CH_2O(PO_3H_2)}\end{array} + \begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{CHOH}\\ |\\ \mathrm{CH_2O(PO_3H_2)}\end{array}$$

Dihydroxyacetone phosphate 3-Glyceraldehyde phosphate α-Glycerophosphate 3-Phosphoglyceric acid

α-Glycerophosphate is hydrolyzed to glycerol and phosphoric acid:

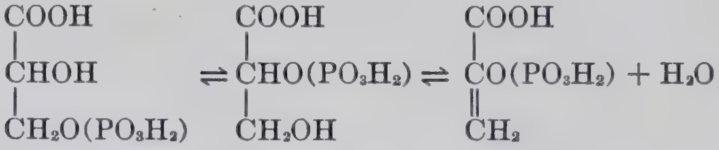


$$\begin{array}{l}\mathrm{CH_2OH}\\ |\\ \mathrm{CHOH}\\ |\\ \mathrm{CH_2O(PO_3H_2)}\end{array} \xrightarrow{+\mathrm{H_2O}} \begin{array}{l}\mathrm{CH_2OH}\\ |\\ \mathrm{CHOH}\\ |\\ \mathrm{CH_2OH}\end{array} + \mathrm{H_3PO_4}$$

α-Glycerophosphate Glycerol Phosphoric acid

The preceding reaction accounts for the small amount of glycerol t is produced in the normal ethyl alcohol fermentation.

3-Phosphoglyceric acid proceeds, through a series of reversible enzy reactions, to break down through 2-phosphoglyceric acid to phosp pyruvic acid:

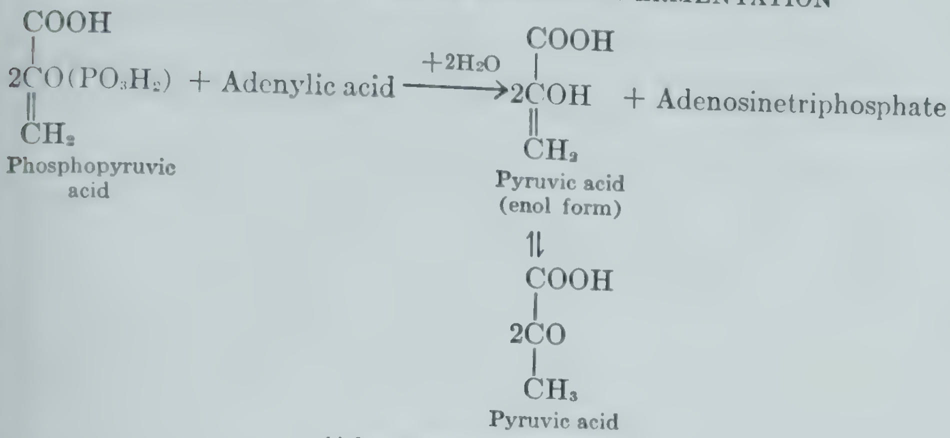


$$\begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{CHOH}\\ |\\ \mathrm{CH_2O(PO_3H_2)}\end{array} \rightleftharpoons \begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{CHO(PO_3H_2)}\\ |\\ \mathrm{CH_2OH}\end{array} \rightleftharpoons \begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{CO(PO_3H_2)}\\ \|\\ \mathrm{CH_2}\end{array} + \mathrm{H_2O}$$

3-Phosphoglyceric acid 2-Phosphoglyceric acid Phosphopyruvic acid

The addition of sodium fluoride to a yeast extract containing eit 3-phosphoglyceric acid or 2-phosphoglyceric acid prevents the formati of phosphopyruvic acid, for the enzyme (enolase) is poisoned.

Phosphopyruvic acid is dephosphorylated by adenylic acid to fo pyruvic acid, the adenylic acid taking up phosphate to become ade sinetriphosphate.



$$\underset{\text{Phosphopyruvic acid}}{\begin{array}{c}COOH\\|\\2CO(PO_3H_2)\\\|\\CH_2\end{array}} + \text{Adenylic acid} \xrightarrow{+2H_2O} \underset{\text{Pyruvic acid (enol form)}}{\begin{array}{c}COOH\\|\\2COH\\\|\\CH_2\end{array}} + \text{Adenosinetriphosphate}$$

$$\rightleftarrows \quad \underset{\text{Pyruvic acid}}{\begin{array}{c}COOH\\|\\2CO\\|\\CH_3\end{array}}$$

(Adenylic system reaction)

denosinetriphosphate donates phosphate to 2 molecules of hexose to n 2 molecules of hexosemonophosphate and 1 molecule of adenylic !, the net result being expressed by the following reaction:

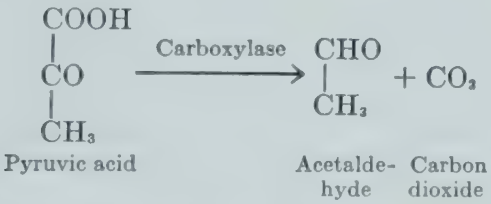
Phosphopyruvic acid + Hexose → Pyruvic acid + Hexosemonophosphate

dditional phosphopyruvic acid is broken down in the same manner ı the result that the hexosemonophosphate is further phosphorylated:

phopyruvic acid + Hexosemonophosphate → Pyruvic acid + Hexosediphosphate

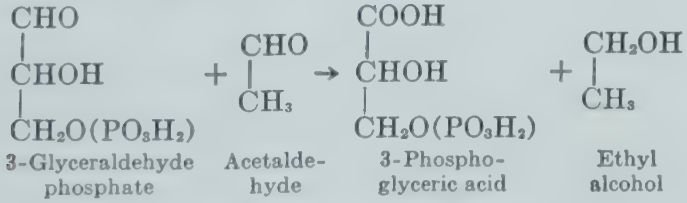
ı this manner, hexosediphosphate arises during the stationary phase ermentation.

yruvic acid is broken down to acetaldehyde and carbon dioxide by enzyme carboxylase:



$$\underset{\text{Pyruvic acid}}{\begin{array}{c}COOH\\|\\CO\\|\\CH_3\end{array}} \xrightarrow{\text{Carboxylase}} \underset{\text{Acetaldehyde}}{\begin{array}{c}CHO\\|\\CH_3\end{array}} + \underset{\text{Carbon dioxide}}{CO_2}$$

hen present in sufficient quantity, acetaldehyde reacts with reduced mase (coenzyme I) with the result that acetaldehyde is reduced to l alcohol while cozymase is oxidized. The oxidized cozymase now s with the triosephosphate (glyceraldehyde phosphate), oxidizing it phosphoglyceric acid, while cozymase becomes reduced again. It is seen that cozymase acts in the capacity of a hydrogen carrier in oxidation-reduction. This reaction occurs normally during the staary phase of fermentation.



$$\underset{\text{3-Glyceraldehyde phosphate}}{\begin{array}{c}CHO\\|\\CHOH\\|\\CH_2O(PO_3H_2)\end{array}} + \underset{\text{Acetaldehyde}}{\begin{array}{c}CHO\\|\\CH_3\end{array}} \rightarrow \underset{\text{3-Phosphoglyceric acid}}{\begin{array}{c}COOH\\|\\CHOH\\|\\CH_2O(PO_3H_2)\end{array}} + \underset{\text{Ethyl alcohol}}{\begin{array}{c}CH_2OH\\|\\CH_3\end{array}}$$

(Cozymase oxidation-reduction reaction)

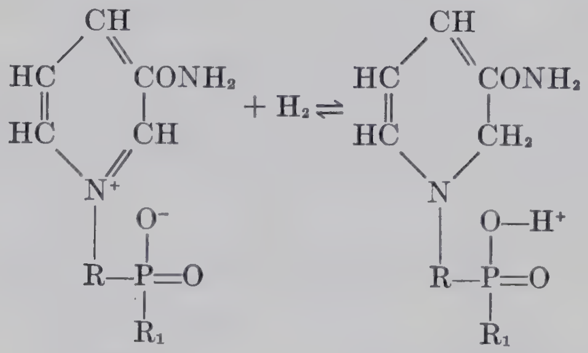
ıe phosphoglyceric acid thus formed is broken down through the s of reactions outlined above to produce more acetaldehyde, which reacts with more triosephosphate to produce more phosphoglyceric The cycle continues in this manner to the end of the fermentation.

**Types of Reactions.** Meyerhof recognizes four main types of tions in the foregoing scheme for the intermediate reactions of the alcohol fermentation. These include the phosphorylation-dephosph ation reactions, the oxidation-reduction reactions, the reversible tions, and the decarboxylation reaction.

The adenylic system, the mechanism of which has already bee plained, is concerned in the phosphorylation-dephosphorylation reac

Oxidoreductases and cozymase (coenzyme I) are responsible fo conversion of the triosephosphates to α-glycerophosphoric acid 3-phosphoglyceric acid during the induction period and for the oxid of triosephosphate (glyceraldehyde phosphate) with the simulta reduction of acetaldehyde during the stationary period of fermenta

Nicotinic acid amide is the hydrogen-carrying group of cozymase. function of cozymase in transporting hydrogen may be illustrated by following equation, in which R represents the ribose phosphoric group of cozymase and $R_1$ represents the adenosinemonophosp group:



Cozymase

Reduced cozymase
(Dihydrocozymase)

The principal reversible reactions include the reaction between hex diphosphate and 2 molecules of triosephosphate; the reaction betw dihydroxyacetone phosphate and glyceraldehyde phosphate; and reactions between 3-phosphoglyceric acid, 2-phosphoglyceric acid, phosphopyruvic acid.

A summary of the reversible reactions appears in the scheme of Me hof shown on page 141. The relation of each intermediate or end pro to its immediate predecessor is indicated.

**Some Enzymes Active during Fermentation.** Several enzymes c lyze the intermediate reactions of the ethyl alcohol fermentation. He kinase converts fermentable hexoses to more active forms. Zymohex catalyzes the breakdown of hexosediphosphate to triosephosphates. Oxi reductase (mutase, dehydrase) with cozymase plays a very import role in the oxidation-reduction reactions. The conversion of phosp pyruvic acid to pyruvic and phosphoric acids is catalyzed by enol while carboxylase breaks down pyruvic acid to form acetaldehyde carbon dioxide.

In Table 41 (Cori, 1942) are shown reactions catalyzed by 12 sepa

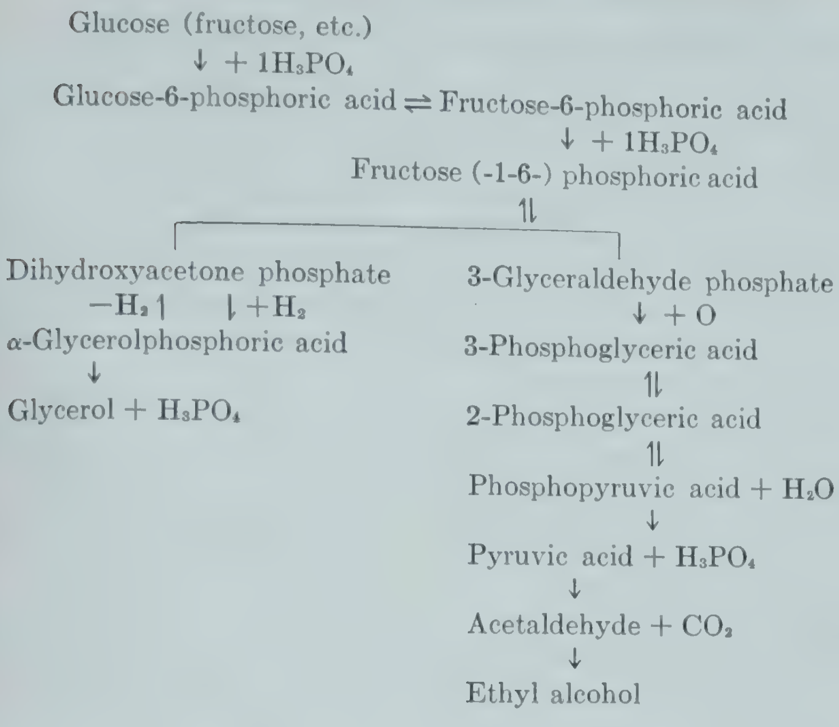

Glucose (fructose, etc.)
$\downarrow + 1H_3PO_4$
Glucose-6-phosphoric acid ⇌ Fructose-6-phosphoric acid
$\downarrow + 1H_3PO_4$
Fructose (-1-6-) phosphoric acid
Dihydroxyacetone phosphate
$-H_2$
$+H_2$
α-Glycerolphosphoric acid
Glycerol + $H_3PO_4$
3-Glyceraldehyde phosphate
$\downarrow + O$
3-Phosphoglyceric acid
2-Phosphoglyceric acid
Phosphopyruvic acid + $H_2O$
Pyruvic acid + $H_3PO_4$
Acetaldehyde + $CO_2$
Ethyl alcohol

TABLE 41. ALCOHOLIC FERMENTATION [a]

act yeast cell: glucose = $2CO_2$ + 2 alcohol; cell-free juice: 2 glucose + $2KH_2PO_4$ = $2CO_2$ + 2 alcohol + 1 hexosediphosphate)

| dialyzable enzymes and reactions catalyzed | Dialyzable components |
|---|---|
| Glucose + ATP → glucose-6-P + ADP* | *Mg + + ions needed for reaction |
| Glucose-6-P ⇌ fructose-6-P | ATP = Adenosinetriphosphate |
| Fructose-6-P + ATP → fructose-1-6-diP + ADP* | ADP = Adenosinediphosphate |
| Fructose-diP ⇌ dihydroxyacetone-P + glyceraldehyde-P | |
| Dihydroxyacetone-P ⇌ glyceraldehyde-P | |
| 3-Glyceraldehyde-P + $KH_2PO_4$ + Pyr. ⇌ 1-3-glyceric acid-P + $H_2$Pyr.[b] | Pyr. = Pyridine-nucleotide |
| | $H_2$Pyr. = Reduced pyridine-nucleotide |
| -3-Glyceric acid-P + ADP ⇌ 3-glyceric acid-P + ATP* | $KH_2PO_4$ = inorganic phosphate |
| 3-Glyceric acid-P ⇌ 2-glyceric acid P* | |
| 2-Glyceric acid-P ⇌ pyruvic acid-P + $H_2O$* [b] | |
| Pyruvic acid-P + ADP → pyruvic acid + ATP* | |
| Pyruvic acid + DPT → acetaldehyde + $CO_2$ | DPT = Diphosphothiamin |
| Acetaldehyde + $H_2$Pyr. ⇌ alcohol + Pyr.[b] | |

---

TE: Owing to the splitting of the sugar molecules in two halves in reaction 4, ions 6 to 12 should be multiplied by 2 to balance the equation given above for t yeast cells.

. F. Cori, *Proc. 5th Ann. Meet. Am. Soc. Brewing Chemists,* May 25-27, 1942.

nzymes obtained in crystalline form.

enzymes and dialyzable components required for the action of som these enzymes.

**Basis for Theory.** Space permits only a relatively brief discussio the facts upon which the foregoing theory is based. Many of the im tant discoveries were made during investigations of the intermed reactions concerned in the formation of lactic acid in muscle extract were subsequently established for the ethyl alcohol fermentation.

Parnas, Lutwak-Mann, and Mann have demonstrated the importanc adenylic acid and adenosinetriphosphate as dephosphorylating and p phorylating agents, respectively.

Hexosediphosphate has been isolated repeatedly and is readily mented by yeast and muscle extracts.

Hexosediphosphate enters into equilibrium with the 2 molecules of tri phosphate (Meyerhof and Lohmann, 1934): dihydroxyacetone phosph synthesized by Kiessling, and 3-glyceraldehyde phosphate, synthesi by Fischer (1932). These two triosephosphates have been isolated fr muscle extract and subsequently from yeast extract fermentations of g cose or of hexosediphosphate with the aid of monoiodoacetic acid. T are readily fermented by yeast extract and together form an equilibri in which dihydroxyacetone phosphate predominates. Whether one sta with a mixture of synthetic or natural triosephosphates or with hexo diphosphate, one finds that a reversible reaction quickly occurs betw hexosediphosphate and the triosephosphates in the presence of ye extract.

Important information concerning the 3-carbon intermediates has b obtained by the use of sodium fluoride and monoiodoacetic acid. Emb (1933), using a muscle extract poisoned with sodium fluoride, show that phosphoglyceric acid was an intermediate compound. He show that, in the absence of fluoride, phosphoglyceric acid was converted pyruvic and phosphoric acid through enzymic action. Nilsson isola phosphoglyceric acid from a fermentation by yeast extract.

Meyerhof and Kiessling showed that there were two phosphoglyce acids: 3-phosphoglyceric acid and 2-phosphoglyceric acid. These ac have been isolated from muscle extract and synthesized by Kiessli They differ in structure, solubilities, and optical rotations.

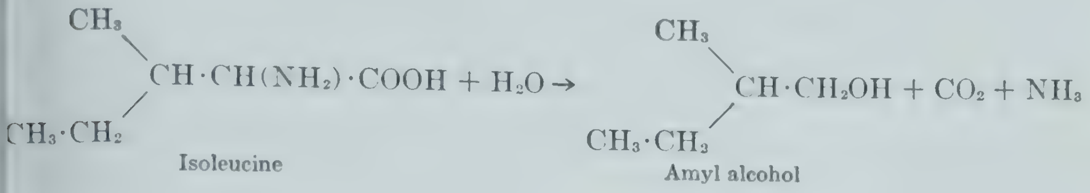
Phosphopyruvic acid was isolated by K. Lohmann as a crystalline s It has been synthesized by Kiessling.

Pyruvic acid has been isolated as an intermediate and is readily f mented by living yeasts or yeast extracts. It is converted to acetal hyde and carbon dioxide by the enzyme carboxylase, discovered Neuberg.

Acetaldehyde has been demonstrated by fixation with sulfites a dimedon.

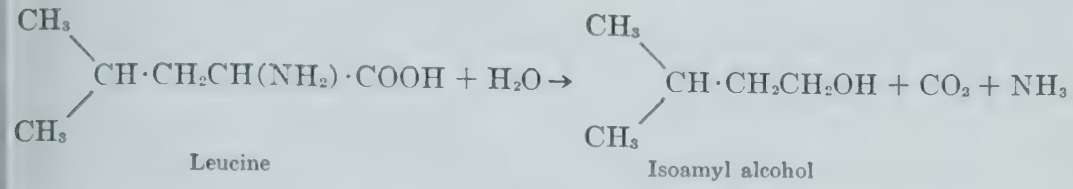
The reader is referred to the reviews of Clifton (1957) and others additional details on this subject.

**The Origin of Amyl and Isoamyl Alcohols.** Ehrlich has shown t amyl and isoamyl alcohols are derived from the amino acids, name oleucine and leucine, respectively. These acids are obtained from the ıedium usually, but in cases of nitrogen deficiency may originate from east protein.



$$\begin{matrix} CH_3 \\ CH_3\cdot CH_2 \end{matrix}\!>\!CH\cdot CH(NH_2)\cdot COOH + H_2O \rightarrow \begin{matrix} CH_3 \\ CH_3\cdot CH_2 \end{matrix}\!>\!CH\cdot CH_2OH + CO_2 + NH_3$$

Isoleucine Amyl alcohol



$$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!>\!CH\cdot CH_2CH(NH_2)\cdot COOH + H_2O \rightarrow \begin{matrix} CH_3 \\ CH_3 \end{matrix}\!>\!CH\cdot CH_2CH_2OH + CO_2 + NH_3$$

Leucine Isoamyl alcohol

The formation of these alcohols from their corresponding amino acids ay be demonstrated by inoculating sterile mashes, containing a fer-entable sugar and a measured quantity of one or both of the acids, ith a pure culture of yeast and analyzing the mashes after the fermen-tion for the amino acids and alcohols. Control experiments should be rried out at the same time, of course, with mashes that do not contain lded amino acids.

The ammonia set free in the foregoing reactions is utilized immediately the yeast cells.

The relative proportions and likewise the quantities of the two alcohols rmed depend on the composition of the medium, i.e., on the relative nounts of the two amino acids, the presence or absence of ammonium lts, and the nature of the sugar; on the species of yeast; on the nutri-e condition of the yeast; and on other factors. Certain ammonium salts use a diminution in the normal yield of amyl alcohols, ammonia being rived from the salts rather than from the amino acids.

Leucine and isoleucine are not converted to their corresponding alco-ls by living yeast cells in the absence of a fermentable sugar, nor are y converted by yeast juice or by zymin (a product prepared by treat-yeast with alcohol and ether, or by acetone and ether).

Alcohols are produced from other $\alpha$-amino acids in a similar manner yeasts. Such products are believed to contribute to the flavor of beer, ne, rum, and other alcoholic beverages. For example, tyrosol, the ohol produced from tyrosine, possesses a bitter taste and is concerned the flavor of beer.

**Succinic Acid ($HOOC\cdot CH_2\cdot CH_2\cdot COOH$).** Succinic acid is believed arise from glutamic acid during fermentation. Ehrlich discovered 909) after experimentation with several amino acids, which included artic acid ($HOOC\cdot CH_2\cdot CH(NH_2)\cdot COOH$), that glutamic acid was only amino acid added to the fermentation medium that produced a ll-defined increase in the quantity of succinic acid.

The probable course of its formation from glutamic acid is as follows:

$$\begin{array}{rl}
\mathrm{HOOC{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH} & \text{(Glutamic acid)} \\
\downarrow \tfrac{1}{2}O_2 & \text{(Oxidative deamination)} \\
\mathrm{NH_3 + HOOC{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}COOH} & (\alpha\text{-Ketoglutaric acid}) \\
\downarrow & \text{(Decarboxylation)} \\
\mathrm{CO_2 + HOOC{\cdot}CH_2{\cdot}CH_2{\cdot}CHO} & \text{(Succinic semialdehyde)} \\
\downarrow \tfrac{1}{2}O_2 & \text{(Oxidation)} \\
\mathrm{HOOC{\cdot}CH_2{\cdot}CH_2{\cdot}COOH} & \text{(Succinic acid)}
\end{array}$$

In the absence of added nitrogen-containing substances, succinic a may be produced from the glutamic acid derived from the autolysis of protein of yeast.

Succinic acid is not formed by yeast in the absence of sugar, nor it produced by yeast juice or zymin.

### References on the Mechanism of the Ethyl Alcohol Fermentation

Anderson, C. G.: "An Introduction to Bacteriological Chemistry," The Williams Wilkins Company, Baltimore, 1938.

Anhagen, E.: Co-Carboxylase, ein neues Co-Enzym der alkoholischen Gärun *Zeit. physiol. Chem.*, **204**: 149 (1932).

Clifton, C. E.: "Introduction to Bacterial Physiology," McGraw-Hill Book Compan Inc., New York, 1957.

Cori, C. F.: The Enzymatic Reactions in Alcoholic Fermentation, *Proc. 5th A Meet. Am. Soc. Brewing Chemists,* May 25–27, 1942.

Embden, G., H. J. Deuticke, und G. Graft: Über die intermediären Vorgänge der Glykolyse in der Muskulatur, *Klin. Wochschr.*, **12**: 213 (1932).

Euler, H. von: Neure Ergebnisse an enzymatischen Oxydations- und Reduktio systemen, *Ergeb. Enzymforsch.*, **3**: 135 (1934).

——— und K. Myrbäck: Co-Zymase und Adenylsäure, *Zeit. physiol. Chem.*, **19** 189 (1931).

——— und R. Nilsson: Adenosintriphosphorsäure und Co-Zymase, *Zeit. physi Chem.*, **208**: 173 (1932).

Fischer, H. O. L., und E. Baer: Über die 3-Glycerinaldehyde-phosphorsäure, *Be* **65**: 337 (1932).

Frey, C. N.: History and Development of Modern Yeast Industry, *Ind. Eng. Chem* **22**: 1154 (1930).

Goda, T.: Beitrag zur enzymatischen Oxydoreduktion zwischen Glycerinphospho säure und Brenztraubensäure, *Biochem. Zeit.*, **297**: 347 (1938).

———: Kann lebende Hefe Hexosen direkt vergären? *Biochem. Zeit.*, **298**: 4 (1938).

Harden, A.: "Alcoholic Fermentation," 4th ed., Longmans, Green & Co., Inc., Ne York, 1932.

——— and R. Robison: A New Phosphoric Ester Obtained by the Aid of Yeas juice (Preliminary Note), *Proc. Chem. Soc.*, **30**: 16 (1914).

——— and W. J. Young: The Influence of Phosphates on the Fermentation Glycose by Yeast Juice (Preliminary Communication), *Proc. Chem. Soc.*, **21**: 1 (1905).

Hopkins, R. H., and C. Krause: "Biochemistry Applied to Malting and Brewing D. Van Nostrand Company, Inc., Princeton, N.J., 1937.

Kiessling, W.: Über die Synthese der Dioxy-aceton-phosphorsäure, *Ber.*, **67**: 8 (1934).

———: Über die Reindarstellung von Glucose-1-phosphorsäure (Cori-Ester), *Bio chem. Zeit.*, **298**: 421 (1938).

——— und O. Meyerhof: Über ein Adenindinucleotid der Hefe: Di-(Andenosin-5 phosphorsäure), *Biochem. Zeit.*, **298**: 410 (1938).

Kluyver, A. J.: "The Chemical Activities of Micro-organisms," University of Londo Press, London, 1931.

——: Die bakteriellen Zuckervergärungen, *Ergeb. Enzymforsch.*, **4**: 230 (1935).

edev, A.: Sur l'extraction de la zymase, *Compt. rend.*, **152**: 1129 (1911).

mann, J.: Aktivierung von Hefealkoholdehydrogenase durch Co-Enzym, *Biochem. Zeit.*, **272**: 95 (1934).

——: Zur Kenntnis biologischer Oxydations-Reduktions-potentiale. Vorläufige Potentialmessungen im System. Alkohol-Dehydrogenase-Acetaldehyd, *Biochem. Zeit.*, **274**: 321 (1934).

——: Über den Mechanismus der Vergärung des Dioxyacetons, *Biochem. Zeit.*, **277**: 261–267 (1935).

vene, P. A., and A. L. Raymond: Hexosemonophosphates. Glucose-3-Phosphate, Glucose-6-Phosphate and Their Bearing on the Structure of Robison's Ester, *Jour. Biol. Chem.*, **89**: 479 (1930).

—— and ———: Hexosemonophosphate (Robison) Natural and Synthetic, *Jour. Biol. Chem.*, **91**: 751 (1931).

—— and ———: Hexosemonophosphates. Synthetic Robison Ester, *Jour. Biol. Chem.*, **92**: 757 (1931).

—— and ———: Hexosemonophosphates. Galactose-6-Phosphate, *Jour. Biol. Chem.*, **92**: 765 (1931).

hmann, K.: Über die Isolierung verschiedener natürlicher Phosphorsäureverbindungen und die Frage ihrer Einheitlichkeit, *Biochem. Zeit.*, **294**: 306 (1928).

—— und P. Schuster: Untersuchungen über die Co-carboxylase, *Biochem. Zeit.*, **294**: 188 (1937).

—— und ———: Über die Co-carboxylase, *Naturwissenschaften*, **25**: 26 (1937).

twak-Mann, C., und T. Mann: Über die Verkettung der chemischen Umsetzungen in der alkoholischen Gärung, I, *Biochem. Zeit.*, **281**: 140 (1935).

yerhof, O.: Intermediate Products and the Last Stages of Carbohydrate Breakdown in the Metabolism of the Muscle and in Alcohol Fermentation, *Nature*, **132**: 337, 373 (1933).

——: Sur les processus intermédiares dans la dégradation des glucides (formation d'acid lactique et fermentation alcoolique), *Ann. inst. Pasteur.*, **53**: 221 (1934).

——: Über die Intermediärvorgänge bei der biologischen Kohlenhydratspaltung, *Ergeb. Enzymforsch.*, **4**: 208 (1935).

——: The Intermediary Reactions of Fermentation, *Nature*, **141**: 855–858 (1938).

——: Intermediate Reactions of Fermentation, *Wallerstein Labs. Communs.*, **5**(16): 181–186 (1942).

——: New Chemical and Physical Tools for Investigating the Intermediary Metabolism of Carbohydrate, *Wallerstein Labs. Communs.*, **6**(17): 19–25 (1943).

——: Enzymatic Mechanisms of Fermentation, *Am. Brewer*, January, 1943.

——: The Origin of the Reaction of Harden and Young in Cell-free Alcoholic Fermentation, *Jour. Biol. Chem.*, **157**(1): 105–119 (1945).

——: New Investigations in the Kinetics of Cell-free Alcoholic Fermentation, *Antonie Van Leeuwenhoek Jour. Microbiol. Serol.*, **12**: 140–144 (1947) (Jubilee Vol. A. J. Kluyver).

—— und W. Kiessling: Über das Auftreten und den Umsatz der α-Glycerinphosphorsäure bei der enzymatischen Kohlenhydratspaltung, *Biochem. Zeit.*, **264**: 40 (1933).

—— und ———: Über die phosphorylierten Zwischen-produkte und die letzten Phasen der alkoholischen Gärung, *Biochem. Zeit.*, **267**: 313–348 (1933).

—— und ———: Über die Isolierung der isomeren Phosphoglycerinsäuren (Glycerinsäure-2-Phosphorsäure und Glycerinsäure-3-Phosphorsäure) aus Gäransätzen und ihr enzymatisches Gleichgewicht, *Biochem. Zeit.*, **276**: 239 (1935).

—— und ———: Über die enzymatische Umwandlung von Glycerinaldehydphosphorsäure in Dioxyacetonphosphorsäure, *Biochem. Zeit.*, **279**: 40 (1935).

—— und ———: Über die enzymatische Umsatz der synthetischen Phosphobrenztraubensäure (enol-Brenztraubensäure-phosphorsäure), $CH_2{=}C(OH_2PO_3)\cdot$COOH, *Biochem. Zeit.*, **280**: 99 (1935).

—— und ———: Die Umesterungsreaktion der Phosphobrenztraubensäure bei der alkoholischen Zuckergärung, *Biochem. Zeit.*, **281**: 249 (1935).

——— und ———: Über den Hauptweg der Milchsäurebildung in der Musku *Biochem. Zeit.*, **283**: 83 (1935).

——— und K. Lohmann: Über den Nachweis von Triosephosphorsäure als Zwis produkt bei der enzymatischen Kohlenhydratspaltung, *Naturwissenschaften* 134 (1934).

——— und ———: Über die enzymatische Gleichgewichts-reaktion zwischen He diphosphorsäure und Dioxyacetonphosphorsäure, *Naturwissenschaften*, **22**: (1934).

——— und ———: Über die enzymatische Gleichgewichts-reaktion zwischen Hex diphosphorsäure und Dioxyacetonphosphorsäure, *Biochem. Zeit.*, **271**: 89 (1

——— und ———: Über die enzymatische Umwandlung von Phosphoglycerins in Brenztraubensäure und Phosphorsäure, *Biochem. Zeit.*, **273**: 60 (1934).

——— und ———: Über die enzymatische Gleichgewichts-reaktion zwischen Hex diphosphorsäure und Dioxyacetonphosphorsäure. II. Die negative Wär tönung der enzymatischen Spaltung, *Biochem. Zeit.*, **273**: 73 (1934).

——— und ———: Über die enzymatische Gleichgewichts-reaktion zwischen Hex diphosphorsäure und Dioxyacetonphosphorsäure. III. Über Abfangen der Tri phosphorsäure mit Bisulfit und die Verbreitung des Ferments "Zymohexase" den verschiedenen Zellarten, *Biochem. Zeit.*, **273**: 413 (1934).

——— und ———: Über die enzymatische Gleichgewichts-reaktion zwischen Hex diphosphorsäure und Dioxyacetonphosphorsäure. IV. (Berichtigung zur II. M teilung), *Biochem. Zeit.*, **275**: 430 (1935).

———, ———, und P. Schuster: Über die Aldolase ein Kohlenstoffverknüpfen Ferment. Aldokondensation von Dioxyacetonphosphorsäure mit Acetaldeh *Biochem. Zeit.*, **286**: 301, 319 (1936).

——— and P. Oesper: The Mechanism of the Oxidative Reaction in Fermentati *Jour. Biol. Chem.*, **170**(1): 1–22 (1947).

———, P. Ohlmeyer, W. Gentner, und H. Meir-Leibnitz: Studium der Zwische reaktionen der Glykolyse mit Hilfe von radioaktivem Phosphor, *Biochem. Ze* **298**: 396 (1938).

———, ———, und W. Möhle: Über die Koppelung zwischen Oxydoreduktion u Phosphatveresterung bei der anaeroben Kohlenhydratspaltung, *Biochem. Ze* **297**: 90 (1938).

——— und W. Shulz: Eine neue Bestimmungsmethode der Phosphoglycerinsäu *Biochem. Zeit.*, **297**: 60 (1938).

Michaelis, L.: Chemistry of Alcoholic Fermentation, *Ind. Eng. Chem.*, **27**: 10 (1935).

———, V. Moraques-Gonzales, and C. V. Smythe: Action of various dyestuffs fermentation and phosphate synthesis in yeast extract, *Enzymologia*, **3**: 2 (1937).

——— and C. V. Smythe: Influence of Certain Dyestuffs on Fermentation and Resp ration of Yeast Extract, *Jour. Biol. Chem.*, **113**: 717 (1936).

Myrbäck, K.: Die Co-Zymase und ihre Bestimmung, *Zeit. physiol. Chem.*, **177**: 1 (1928).

———: Co-Zymase, *Ergeb. Enzymforsch.*, **2**: 139 (1933).

———: Zur Kenntnis der Co-Zymase, V, *Zeit. physiol. Chem.*, **233**: 95 (1935).

——— und H. von Euler: Gärungs-Co-Enzym (Co-Zymase) der Hefe, IV, *Ze physiol. Chem.*, **138**: 1 (1924).

——— und ———: Co-Zymase und die Aktivierung der Gärung frischer Hefe dur Hefenextrakten, *Zeit. physiol. Chem.*, **176**: 258 (1928).

——— und B. Örtenblad: Zur Kenntnis der Co-Zymase, IV, *Zeit. physiol. Chem* **233**: 87 (1935).

Neuberg, C.: Development of Chemistry of Fermentation, *Am. Brewer*, **75**: 22– (May, 1942).

——— und A. Hildesheimer: Über zuckerfreie Hefegärungen, I, *Biochem. Zeit.*, 3 170 (1911).

——— und J. Hirsch: Öber den Verlauf der alkoholischen-Gärung bei alkalisch Reaktion. II. Gärung mit lebender Hefe in alkalischen Lösungen, *Biochem Zeit.*, **96**: 175 (1919).

—— und ———: Wirkungsweise der Abfangmethode bei der Acetaldehyd-Glycerin-Spaltung des Zuckers, *Biochem. Zeit.*, **98**: 141 (1919).

—— und M. Kobel: Die desmolytisches Bildung von Methylglyoxal durch Hefenenzym, *Biochem. Zeit.*, **203**: 463 (1928).

—— und ———: Die Bildung von Brenztraubensäure als Durchgangsglied bei der alkoholischen Zückerspaltung. Ihre Isolierung als Hauptprodukt der Gärung, *Biochem. Zeit.*, **216**: 493 (1929).

—— und E. Reinfurth: Die Festlegung der Aldehydstufe bei der alkoholischen Gärung. Ein experimenteller Beweis der Acetaldehyd-Brenztraubensäure-Theorie, *Biochem. Zeit.*, **89**: 365 (1918).

lsson, R.: Einige Betrachtungen über den glykolytischen Kohlenhydratabbau, *Biochem. Zeit.*, **258**: 198 (1933).

—— und H. von Euler: Co-Zymase und Adenosintriphosphat, *Zeit. physiol. Chem.*, **204**: 204 (1932).

lmeyer, P.: Über die Gargeschwindigkeit der Hexosediphosphorsäure und ihre Umesterung mit Adenylsäure, *Biochem. Zeit.*, **283**: 114–127 (1935).

——: Darstellung von reiner Dihydrocozymase in Substanz, *Biochem. Zeit.*, **297**: 66 (1938).

tern, P., und A. J. Guthke: Über die Darstellung der Phosphoglycerinsäure, *Zeit. physiol. Chem.*, **248**: 155 (1937).

rnas, J. K.: "Handbuch der Enzymologie," Leipzig, 1940, Vol. II.

——, C. Lutwak-Mann, und T. Mann: Über die Verkeltung der chemischen Umsetzungen in der alkoholischen Gärung, *Biochem. Zeit.*, **281**: 168 (1935).

bison, R.: A New Phosphoric Ester Produced by the Action of Yeast Juice on Hexoses, *Biochem. Jour.*, **16**: 809–824 (1922).

—— and E. J. King: Hexosemonophosphoric Esters, *Biochem. Jour.*, **25**: 323 (1931).

ythe, C. V.: Phosphoric Acid Esters from Yeast Extract, *Jour. Biol. Chem.*, **117**: 135 (1937).

——: An Improved Method of Preparing Hexosemonophosphate from Yeast Extract, *Jour. Biol. Chem.*, **118**: 619 (1937).

phenson, M.: "Bacterial Metabolism," 2d ed., Longmans, Green & Co., Inc., New York, 1939.

rburg, O., und W. Christian: Über ein neues Oxydationsferment und sein Absorptionsspektrum, *Biochem. Zeit.*, **254**: 438 (1932).

## CHAPTER 6

# BREWING

"Brewing," or the production of malt beverages, is the name give the combined processes of preparing beverages from infusions of gr that have undergone sprouting (malting), and the fermenting of sugary solution by yeast, whereby a portion of the carbohydrate changed to alcohol and carbon dioxide. It is an ancient industry and probably invented by the Egyptians.

The term "malt beverage," according to the Federal Alcohol Admi tration Act[1] of May, 1937,

> . . . means a beverage made by the alcoholic fermentation of an infusion decoction, or combination of both, in potable brewing water, of malted ba with hops, or their parts, or their products, and with or without other ma cereals, and with or without the addition of unmalted or prepared cereals, o carbohydrates or products prepared therefrom, and with or without the addi of carbon dioxide, and with or without other wholesome products suitable human food consumption.

Beer, ale, porter, and stout are examples of malt beverages.

**Composition of Beers.** The substances found in a beer will dep largely upon the nature and quality of the raw materials, the treatment the sprouted grain or malt used in mashing, and the character of ensuing fermentation, but storage and finishing operations will like affect the final composition.

In a normal beer one may expect to find carbohydrates—such as d trin, maltose, and glucose—and protein derivatives—such as pepto amino acids, and amides—these products arising mainly as the result the action of the enzymes of the malt. Hops contribute bitter substan resins, essential oil, and tannic acid, but a portion of some of these s stances is lost during the subsequent brewing operations. As a result the alcoholic fermentation, the sugars of the wort are converted, in p at least, to ethanol and carbon dioxide, with much smaller quantities glycerol and acetic acid; while some of the amino acids are transform to higher alcohols and acids, for example, succinic acid. Lactic acid m be added to the wort or may accumulate as the result of the action lactic acid bacteria. Salts are always found and usually traces of The finished beer contains 85 to 92 per cent water by volume. Thu beer is not a simple beverage but one that is capable of wide variat composition unless the conditions of brewing are very carefully ulated.

---

[1] U.S. Department of the Treasury, Federal Alcohol Administration, Federal Adm istration Act as in effect on May 15, 1937.

'able 42 presents the analyses of some malt beverages, while Table 43 ws the range in composition of substrate wort and packaged lager beer.

TABLE 42. ANALYSES OF SOME MALT BEVERAGES *

| Variety | No. of analyses | Specific gravity | Water | Carbonic acid | Alcohol by weight | Extract | Nitrogenous substances | Sugar as maltose | Gum and dextrin | Acid as lactic | Glycerine | Ash | Phosphoric acid |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| nk | 205 | 1.0114 | 91.11 | 0.197 | 3.36 | 5.34 | 0.74 | 0.95 | 3.11 | 0.156 | 0.12 | 0.204 | 0.055 |
| r | 258 | 1.0162 | 90.08 | 0.196 | 3.93 | 5.79 | 0.71 | 0.88 | 3.73 | 0.151 | 0.165 | 0.228 | 0.077 |
| ort beer | 109 | 1.0176 | 89.01 | 0.209 | 4.40 | 6.38 | 0.74 | 1.20 | 3.47 | 0.161 | 0.154 | 0.247 | 0.074 |
| : | 84 | 1.0213 | 87.87 | 0.234 | 4.69 | 7.21 | 0.73 | 1.81 | 3.97 | 0.165 | 0.176 | 0.263 | 0.089 |
| s bier | 26 | 1.0137 | 91.63 | 0.297 | 2.73 | 5.34 | 0.58 | 1.62 | 2.42 | 0.392 | 0.092 | 0.149 | 0.034 |
| er | 40 | 1.0191 | 88.49 | 0.215 | 4.70 | 6.59 | 0.65 | 2.62 | 3.08 | 0.281 | .... | 0.363 | 0.093 |
|  | 38 | 1.0141 | 89.42 | 0.201 | 4.75 | 5.65 | 0.61 | 1.07 | 1.81 | 0.278 | .... | 0.31 | 0.086 |

leprinted by permission, from "Food Inspection and Analysis," 4th ed. by Leach and Winton, ished by John Wiley & Sons, Inc., New York.

LE 43. RANGE IN COMPOSITION OF SUBSTRATE WORT AND PACKAGED LAGER BEER *

|  | Range in composition of |  |
|---|---|---|
|  | Substrate wort | Packaged lager beer |
| cific gravity at 20°/20°C. | 1.04755– 1.04965 | 1.01071–1.01410 |
| ract, ° Plato | 11.80 –12.30 |  |
| act apparent, ° Plato | ................ | 2.74 –3.60 |
| act real, ° Plato (calculated) | ................ | 4.08 –5.45 |
| ıcing sugar, as maltose, % | 7.0 – 8.5 | 0.90 –1.55 |
| r degree, % reducing sugar | 65.0 –72.0 |  |
| hol, % by weight | ................ | 3.10 –3.90 |
|  | 5.2 – 5.8 | 4.10 –4.50 |
| l acidity, as lactic, % | 0.11 – 0.12 | 0.13 –0.17 |
| r, 0.5-in. cell Lovibond series 52 | 3.0 – 5.0 | 2.50 –3.50 |
| ein (N × 6.25), % | 0.38 – 0.50 | 0.24 –0.38 |
| on dioxide, % by weight | ................ | 0.50 –0.57 |

l. Schneider, *Jour. Agr. Food Chem.*, 1: 241–245 (1953). Copyright 1953 by the rican Chemical Society and reprinted by permission of the copyright owner.

**xtent of Manufacture.** Some idea of the magnitude of the industry be gained by noting the data provided in Table 44, which gives the -beverage production for the nine leading states in the United States he Government fiscal years 1936, 1946, and 1956 and the number of veries operated.

**he Brewing Process.** The important steps in the manufacture of or ale include the following: cleaning, grinding, and weighing of the ; grinding (if this is necessary) and weighing of the malt adjuncts; hing of the malt and processing of the malt adjuncts; filtering of the

TABLE 44. MALT-BEVERAGE PRODUCTION BY LEADING STATES DURING FISCAL YEARS 1936, 1946, AND 1956 *

| State | Production, barrels | | | Premises opera | | |
|---|---|---|---|---|---|---|
| | 1936 | 1946 | 1956 | 1936 | 1946 | 19 |
| New York | 8,598,081 | 13,464,043 | 12,001,451 | 70 | 44 | 2 |
| Wisconsin | 5,736,534 | 9,054,093 | 11,446,855 | 91 | 61 | 4 |
| New Jersey | 2.871.205 | 5.230.239 | 7,717,102 | 20 | 13 | |
| Pennsylvania | 6.010,171 | 9,558,788 | 7,694.406 | 112 | 63 | 3 |
| California | 2,448,131 | 4.789,651 | 6,983.711 | 43 | 22 | 1 |
| Missouri | 2,705.133 | 5,858.202 | 6,897.351 | 21 | 13 | |
| Michigan | 3,249,355 | 4.126.082 | 5,374,074 | 52 | 24 | 1 |
| Ohio | 3.432.921 | 5,917.205 | 5,102.073 | 54 | 39 | 1 |
| Illinois | 3,578,180 | 5.968,439 | 3,402.109 | 61 | 39 | 2 |
| Total, United States | 51,812,062 | 84.977,700 | 90,697,911 | 732 | 471 | 28 |

* Data obtained from U.S. Department of the Treasury, Internal Revenue Servic Alcohol and Tobacco Tax Division. *Annual Reports of the Commissioner,* for th fiscal years ended June 30, 1936, 1946, and 1956, respectively.

mashed materials to separate the soluble material (the wort) from th insoluble material (the spent grain); boiling of the wort with hops; separation of the wort from hop residues and protein precipitates; cooling o the wort; fermentation of the wort; clarification, attenuation, and maturing of the malt beverage during storage; chillproofing and carbonation filtration; packaging; pasteurizing; labeling; inspecting; packing; an stamping of the cases.

It will be helpful to the reader to make frequent reference to Figs. 2 to 28 in following the description of the brewing process. Figure 27 give a time-temperature chart of the brewhouse operations, which should b particularly informative. The temperature in this chart is given in term of the Reaumur scale or thermometer on which the freezing point is 0°R and the boiling point is 80°R.

**Raw Materials.** The materials used in making malt beverages includ malt, malt adjuncts, hops, and water. Malt is prepared (usually as a separate operation by malt manufacturers) by soaking selected barley i water, permitting it to germinate, and drying it under carefully regulate conditions.

Although beer may be made from malt, hops, and water in some countries, malt adjuncts are used in the United States owing to the fact tha barleys used for the preparation of malt in this country are richer i protein than barleys used in European countries. A high nitrogen conten is usually undesirable, for it tends to produce a satiating and relativel unstable beer. Usually 65 to 80 per cent malt is used in the United States

In addition to reducing the nitrogen content of the wort, malt adjuncts supply additional carbohydrates to be acted on by the excess of amylases

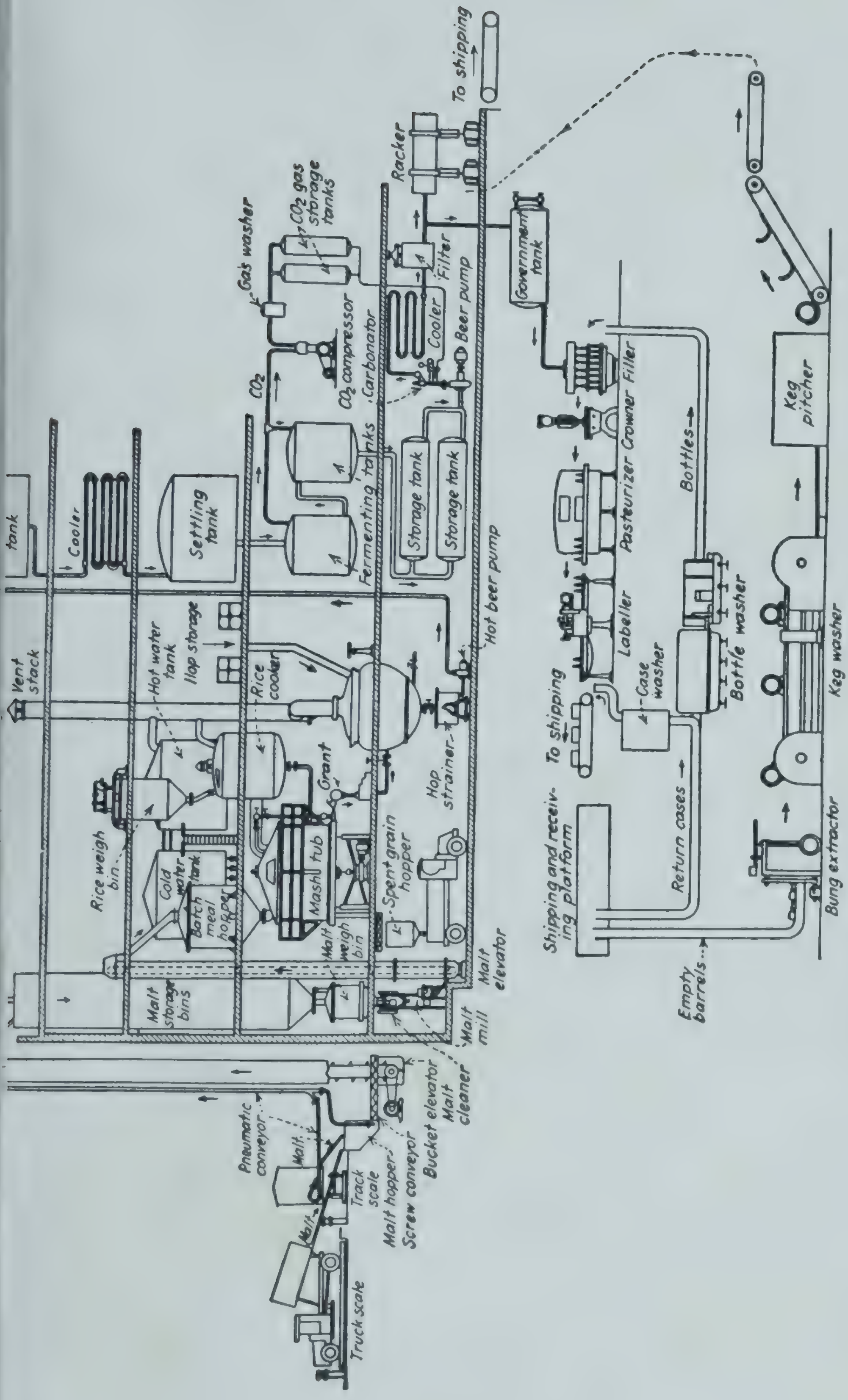

Truck scale
Malt
Malt
Pneumatic conveyor
Track scale
Malt hopper
Screw conveyor
Bucket elevator
Malt cleaner
Malt mill
Malt elevator
Malt storage bins
Malt weigh bin
Spent grain hopper
Batch meal hopper
Cold water tank
Rice weigh bin
Mash tub
Grant
Hop strainer
Hot water tank
Rice cooker
Vent stack
Hop storage
Hot beer pump
tank
Cooler
Settling tank
Fermenting tanks
Storage tank
Storage tank
CO2
CO2 compressor
Carbonator
Gas washer
CO2 gas storage tanks
Cooler
Beer pump
Filter
Racker
To shipping
Government tank
Filler
Crowner
Pasteurizer
Labeller
Case washer
Bottle washer
Bottles
To shipping
Shipping and receiving platform
Return cases
Empty barrels
Bung extractor
Keg washer
Keg pitcher

FIG. 26. Brewery. (Courtesy of L. V. Burton, ed., *Food Inds.*)

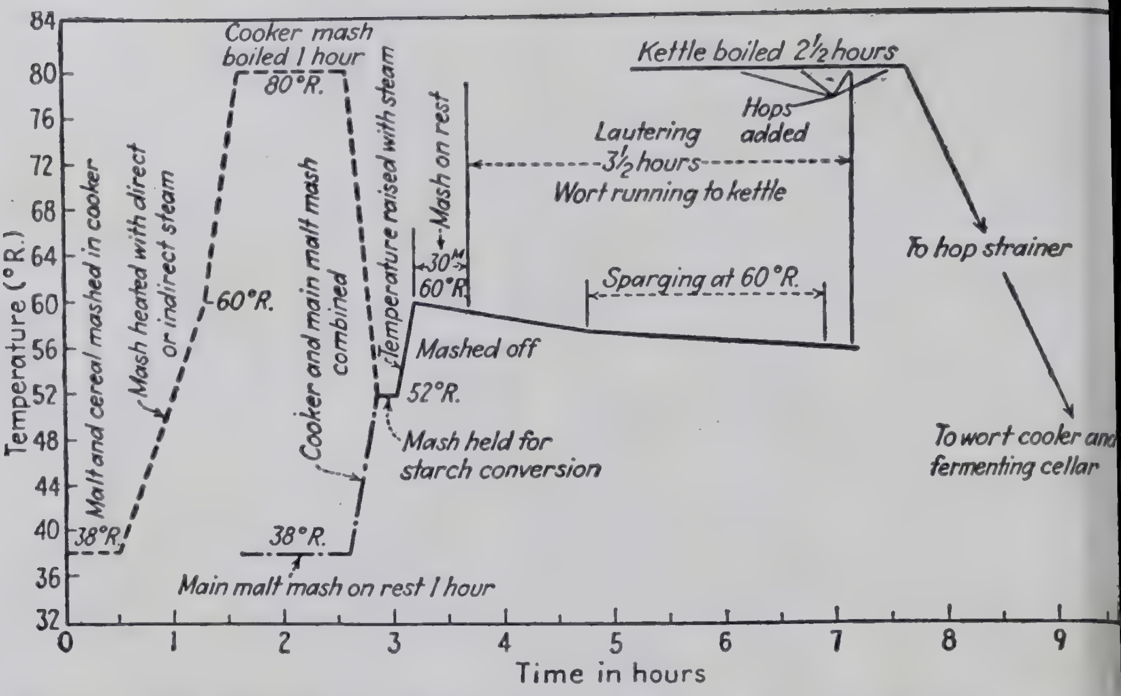


Fig. 27. Time-temperature chart of brewhouse operations. [*Courtesy of R. Schwarz, Ind. Eng. Chem.,* 27: 1031 (1935). *Copyright* 1935 *by the American Chemical Society and reprinted by permission of the copyright owner.*]

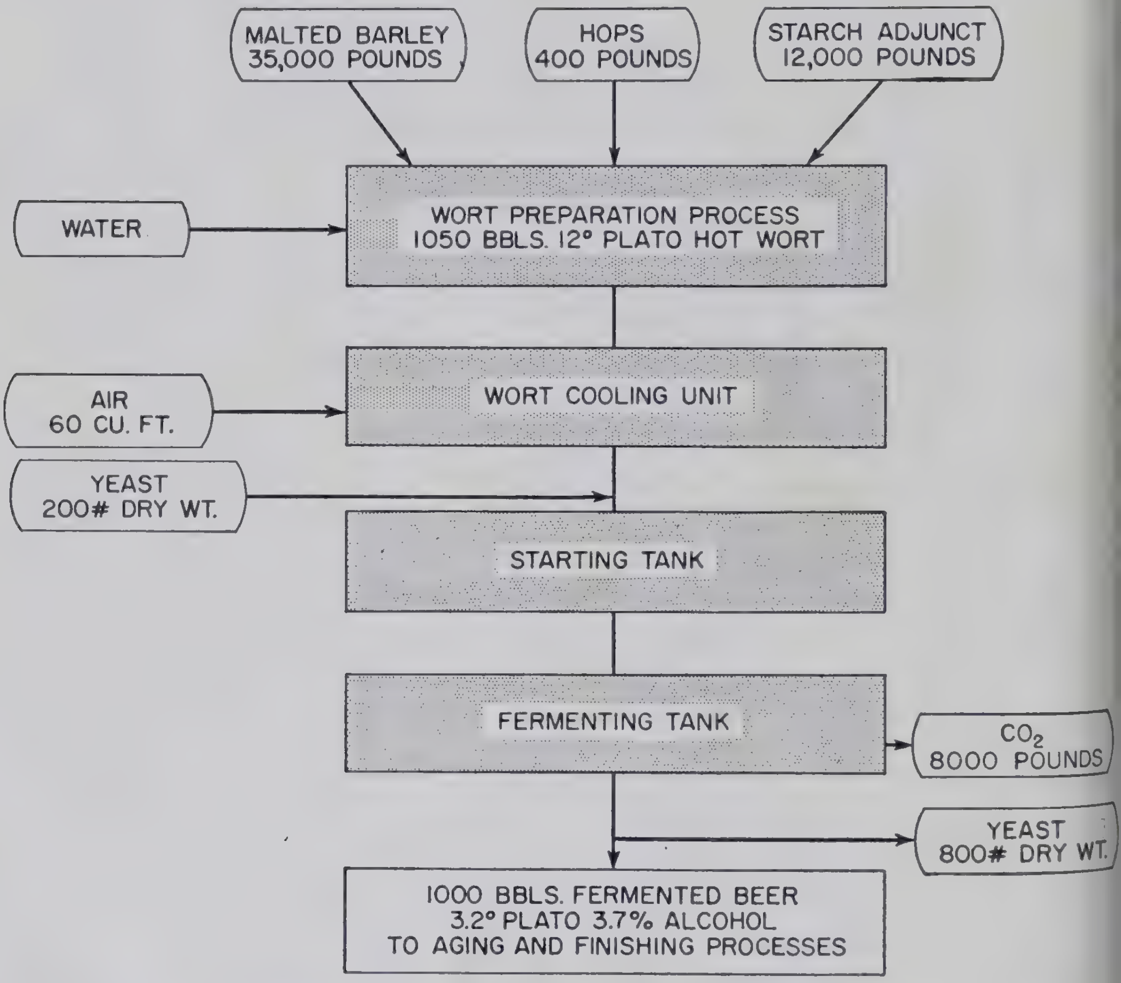

MALTED BARLEY 35,000 POUNDS
HOPS 400 POUNDS
STARCH ADJUNCT 12,000 POUNDS
WATER
WORT PREPARATION PROCESS 1050 BBLS. 12° PLATO HOT WORT
AIR 60 CU. FT.
WORT COOLING UNIT
YEAST 200# DRY WT.
STARTING TANK
FERMENTING TANK
$CO_2$ 8000 POUNDS
YEAST 800# DRY WT.
1000 BBLS. FERMENTED BEER 3.2° PLATO 3.7% ALCOHOL TO AGING AND FINISHING PROCESSES

Fig. 28. Flow diagram for the fermentation of 1,000 barrels of beer. [*Courtesy of R. Schneider, Jour. Agr. Food Chem.,* 1: 241 (1953). *Copyright* 1953 *by the American Chemical Society and reprinted by permission of the copyright owner.*]

resent in the malt and help to produce a beer (or ale) that is less sati-ting, paler in color, and relatively more stable.

Malt adjuncts include certain grains and grain products (cooked or ncooked), sugar and sirups, and miscellaneous other carbohydrate prod-cts. In normal times in this country, corn and corn products (corn grits, orn flakes, and corn sugar), rice, sugar, and sirups are used as the prin-ipal malt adjuncts. With a shortage in malt or the common malt djuncts, other materials may be used, such as wheat, barley, sorghum rain, soybeans and soybean products, cassava and cassava products, nd potatoes and potato products.

The kinds and quantities of materials used in the production of fer-ented malt liquors are illustrated in Table 45.

The quantities of materials used in the production of each barrel of rmented malt liquors are shown in Table 46. These are, of course, aver-ge figures based on nationwide production and are intended to convey nly a broad picture of the malt-beverage industry.

Hop cones (specifically the blossom clusters of the female hop plant) re a source of pyrogallol and catechol tannins, resins, essential oils, and ther constituents. The tannins are important in the precipitation of nstable proteins during the boiling of the wort. The resins, which con-ibute bitterness and biological stability to malt beverages, consist of the o-called alpha and beta resins (soft) and gamma resin (hard) and con-ain humulone, lupulone, and cohumulone.

The essential oils (myrcene, linaloöl, geraniol, humulene, etc.) give he aroma to hops.

Usually 0.4 to 0.7 lb. of hops is used in making a barrel of malt everage in the United States.

**Mashing.** The purpose of mashing is to digest and to dissolve as much possible of the valuable portions of the malt or malt adjuncts.

The sweet wort that results from this enzyme process contains dextrins, altose, other sugars, pentosans, protein degradation products, minerals, nnin, coloring matter, and other substances.

*Mashing Methods.* There are two main methods for mashing: the fusion and decoction methods. Many modifications of these methods e used in practice.

THE INFUSION METHOD. There are two infusion processes: one of these the upward method; the other, the downward method. In the upward ocess, the malt is mixed (doughed in) with water at a temperature of , to 50°C. This main mash is permitted to rest (protein rest period) r about 1 hr. at this temperature to favor the action of the proteolytic zymes. The temperature is then raised to 65 to 70°C. by the doughing of the cooked starchy malt adjuncts, which are at the boiling tem-rature. The mash is permitted to stand at this temperature for a few nutes for starch saccharification. The temperature is then brought to out 75°C., or a little above, for the destruction of the enzymes. The ash is filtered at this temperature.

In the downward infusion process, the initial temperature of the mash ter may be about 77°C. The added malt aids in cooling the water to

TABLE 45. KINDS AND AMOUNTS OF MATERIALS USED IN THE PRODUCTION OF BEER AND CEREAL BEVERAGES BY PRINCIPAL STATES DURING THE FISCAL YEAR 1956 *

| State | Grain and grain products, lb. | | | | | Soybeans and soybean products, lb. | Sugar and sirups, lb. | Hops and hop extracts, lb. |
|---|---|---|---|---|---|---|---|---|
| | Malt | Corn | Rice | Wheat | Total | | | |
| New York | 345,003,150 | 138,076,695 | 45,967,412 | 767,600 | 529,814,857 | 99,825 | 4,413,951 | 4,515,203 |
| Wisconsin | 325,461,521 | 112,569,830 | 71,998,700 | ........ | 510,030,051 | 174,394 | 3,637,069 | 4,191,156 |
| New Jersey | 225,191,333 | 90,668,020 | 28,326,900 | ........ | 344,186,253 | 20,940 | 13,855,784 | 2,802,076 |
| Pennsylvania | 237,299,064 | 76,488,218 | 11,295,679 | 2,423,200 | 327,506,161 | 30,215 | 25,410,791 | 2,948,797 |
| California | 209,510,595 | 40,673,857 | 65,663,505 | ........ | 315,847,957 | 154,161 | 1,868,537 | 2,540,797 |
| Missouri | 206,551,803 | 32,683,495 | 63,794,000 | ........ | 303,029,298 | 45,525 | 6,110,087 | 2,909,056 |
| Michigan | 161,919,097 | 29,004,231 | 49,559,895 | ........ | 240,483,223 | 4,650 | 6,212,426 | 1,823,737 |
| Total, United States | 2,650,652,137 | 871,955,351 | 424,954,817 | 3,679,525 | 3,951,290,530 † | 1,181,776 | 109,404,864 | 32,938,442 ‡ |

* U.S. Department of the Treasury, Internal Revenue Service, Alcohol and Tobacco Tax Division, *Statistics Relating to the Alcohol and Tobacco Industries for the Fiscal Year Ended June* 30, 1956, *Publication* 67.

† Includes 10,000 lb. of barley and barley products and 38,700 lb. of sorghum grain and sorghum-grain products.

‡ Represents 32,924,923 lb. of hops and 13,519 lb. of hop extracts.

BLE 46. AVERAGE QUANTITIES OF MATERIALS USED IN THE PRODUCTION OF 1 BARREL OF FERMENTED MALT LIQUORS *

| Material | Lb./31-gal. barrel | | | Percentage of total material | | |
|---|---|---|---|---|---|---|
| | 1937 | 1946 | 1956 | 1937 | 1946 | 1956 |
| ılt | 37.05 | 25.457 | 29.224 | 71.20 | 56.40 | 64.731 |
| rn and corn products | 7.20 | 9.304 | 9.613 | 13.85 | 20.62 | 21.293 |
| e | 3.92 | 2.834 | 4.685 | 7.55 | 6.28 | 10.377 |
| ıeat | .... | 0.565 | 0.041 | ...... | 1.25 | 0.091 |
| ley | .... | 1.878 | 0.0001 | ...... | 4.16 | 0.0002 |
| ghum grains | .... | 1.842 | 0.0004 | ...... | 4.08 | 0.0009 |
| beans and soybean products | .... | 0.079 | 0.012 | ...... | 0.18 | 0.027 |
| ar and sirups | 3.22 | 2.664 | 1.206 | 6.19 | 5.90 | 2.671 |
| ps and hop extracts | 0.63 | 0.442 | 0.363 | 1.21 | 0.98 | 0.804 |
| ıer materials | .... | 0.065 | ..... | ...... | 0.15 | ...... |
| 'otal material per barrel | 52.02 | 45.130 | 45.147 | 100.00 | 100.00 | 100.000 |
| 'otal malt adjuncts † | 14.34 | 19.231 | 15.560 | 27.59 | 42.62 | 34.465 |

Calculated from statistics relating to the alcohol industries, presented in U.S. ›artment of the Treasury, Internal Revenue Service, Alcohol and Tobacco Tax ision, *Annual Reports of the Commissioner*, for the fiscal years ended June 30, 7, 1946, and 1956.

Total materials less hops, hop extractives, and malt.

›roximately 70°C. A temperature of 65 to 70°C. is maintained, as in upward infusion process, to permit saccharification. The final temature of the mash is lower than the initial temperature. The down'd infusion process is an English method.

HE DECOCTION METHOD. In this method, the mash is mixed at a lower perature, occasionally around 40°C., than is the case with infusion cesses. The temperature of the mash is raised by steps until a final perature of about 75°C. has been obtained.

. portion of the initial mash, approximately one-third, is withdrawn, ted, and boiled for a short period of time and then returned to the n mash. The heated portion raises the temperature of this entire ·h. The enzymes in the boiled portion have been destroyed, but the walls of the grain are softened and the starch liquefied. Diastatic ion is thus facilitated by this process.

'he mash may be maintained for a while at the new temperature. ereafter another portion of the mash may be removed, boiled, and ırned to the main mash. The process may be called a one-, two-, e-mash process or single-, double-, or triple-mashing process, depend- on the number of portions that have been heated and returned to mash.

ı a three-mash process, according to Hopkins and Krause, the mash be held at the initial temperature (40°C.) in order to extract the ymes and favor proteolysis and the action of phytase. The mash may

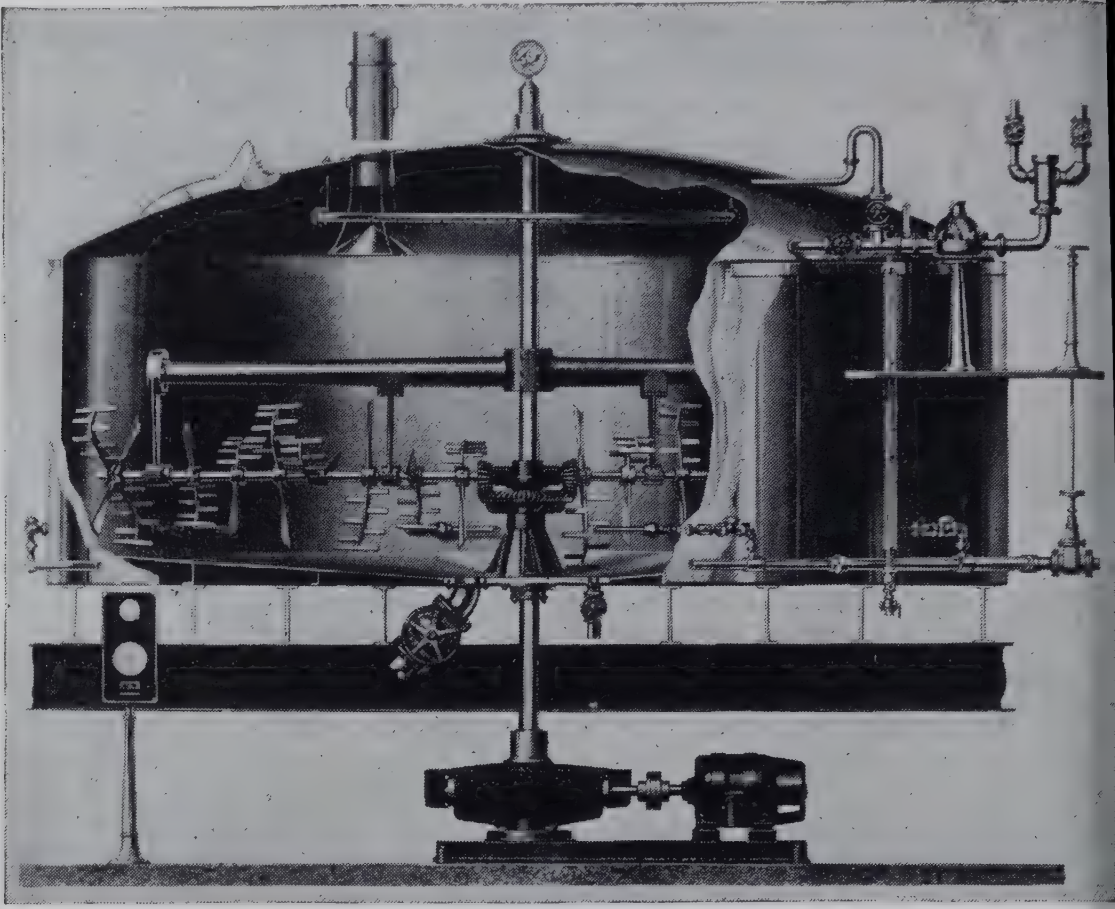


FIG. 29. Vertical mash mixer with conical bottom. (*Courtesy of Schock-Gusmer & Co., Inc., Hoboken, N.J.*)

then be held at 50°C. for "full proteolysis and peptonization," at 60 to 65°C. for saccharification, and at 70 to 75°C. for dextrinization of any unconverted starch by α-amylase and complete extraction.

*Comparison of Methods.* The decoction process usually yields a higher percentage of extract than the infusion process. But the flavor of the wort obtained from the infusion process may be better than that of the decoction process, because bitter resins are not dissolved to such an extent. Either method should produce satisfactory results, however.

*Important Considerations in Mashing.* In mashing, the proper preparation of the malt and uncooked malt adjuncts; the treatment of the mash water, if necessary; and the control of temperature, pH, mash concentration, and time are important.

*Preparation of Malt.* Malt is selected, cleaned, and ground. Grinding, to be satisfactory, must be accomplished according to the variety of malt and the type of equipment being used. There should be proper distribution of the ground malt into husks, coarse grits, fine grits, and flour. The presence of unground kernels results in a loss of extract, while the production of too finely pulverized malt interferes with the filtration of the wort. The properly prepared malt is mixed with a measured quantity of water at the correct temperature in the mash kettle.

*Treatment of Malt Adjuncts.* Unmalted cereals, such as corn meal,

rn grits, rice, or wheat are mixed with water and a small amount of
alt, agitated and brought gradually to a boiling temperature. The
ash, known as a converter mash, is then cooked with or without the use
pressure to gelatinize the starch and render it soluble. The converter
ash is eventually added to the main mash containing the malt.

Malt adjuncts that exist in the prepared state, such as cereal flakes,
e added directly to the main mash without any preliminary cooking.

Sugars and sirups are usually added to the wort.

*Composition of Mash Water.* The composition of the water that is
be used in brewing is very important. The quantity and character of
e dissolved salts are of much significance, while pH is of secondary
terest. Water containing calcium and magnesium sulfates with a per-
anent hardness of between 200 to 300 parts per million (expressed as
lcium carbonate)[1] is usually suitable. On the other hand, soft water
required for the brewing of certain light lager beers. Filtration, clari-
ation, and stability are influenced to some extent by the type of water
ed. "Burtonizing" is the name given to a process of artificially increas-
g the permanent hardness of water for ale and beer making. It is so
lled from Burton-on-Trent in England, where excellent ales have long
en produced and the water supply is regarded as practically perfect
r this purpose. (For a further discussion of this subject, the reader
referred to "Biochemistry Applied to Malting and Brewing," by Hop-
ns and Krause.)

*Temperature.* The temperature of mashing is a very important factor
determining the type of beer to be produced. A high temperature is
ore favorable to the action of α-amylase than of β-amylase. Hence
higher proportion of dextrin will be produced when the mash is con-
ted at a relatively high temperature. The fermentation of a wort
ntaining a relatively large proportion of dextrin results in a low-alcohol
r. On the other hand, the use of a low conversion temperature favors
production of a larger proportion of sugar and less dextrin and, sub-
quently, a high-alcohol beer, or ale.

When the action of α-amylase is favored and that of β-amylase is
ibited, dextrins will accumulate at the expense of maltose. Dextrins
colloidal and, when present in a wort in sufficiently high concentra-
n, will cause a slow filtration of the wort. Sparging will likewise be
icult, and considerable extract will be left in the spent grains. Further-
re, dextrins are not fermented by beer yeasts. It is this fact that
rmits one to control the alcohol in a given beer more readily.

Table 47 illustrates the effect of the temperature of conversion on the
io of sugars to dextrins.

By carefully controlling the temperature, it is thus possible to regulate
relative proportions of sugar to dextrins.

Starch liquefaction is most rapid at 70 to 75°C., while saccharification
ceeds most rapidly at approximately 65°C. The optimum temperature
a given conversion is governed by the pH of the mash, its concentra-
n, and other factors.

---

R. Schwarz, Brewing Processes, *Ind. Eng. Chem.*, 27: 1031 (1935).

TABLE 47. EFFECT OF THE TEMPERATURE OF CONVERSION ON THE RATIO OF SUGA TO DEXTRINS *

| Conversion Temperature, °C. | Ratio of Sugar to Nonsugar |
|---|---|
| 64 | 1:0.37 |
| 66 | 1:0.40 |
| 68 | 1:0.48 |
| 70 | 1:0.52 |
| 72 | 1:0.57 |

* M. A. Pozen, Enzymes in Brewing, *Ind. Eng. Chem.*, **26**: 1127 (1934). Copyrigh 1934 by the American Chemical Society and reprinted by permission of the copyrigh owner.

Temperature very definitely affects the type of proteolysis that occur during mashing. At a conversion temperature of about 60°C., nitrogenous compounds of high molecular weight are formed. These compounds are valuable in the production of palatefulness, foam, and foam stabilization in beer. A greater proportion of amino nitrogen is formed at 50°C. The derivatives of low molecular weight, the amino acids in particular, are useful as food for the yeast in subsequent fermentation. An undue amount of proteolysis, especially the production of amino nitrogen in quantities greatly in excess of those required for the nutrition of the yeast, should be avoided.

*pH.* By properly adjusting the pH of the mash, the quantity of extract will be increased, while subsequent clarification and filtration will be facilitated. The pH of the mash will influence the activity of the enzymes, each of which functions best at a given pH, which is dependent also on the temperature, concentration, and nature of the mash and other factors.

According to Hopkins and Krause, the yield of extract in a mash is maximum at a pH of around 5 to 5.2. β-Amylase is very active at this pH as well as protease. Maltose formation and attenuation are best at a pH of approximately 5.5. This value is excellent for filtration also. At pH values below 5, amino nitrogen is formed at a maximum rate.

Tannin and bitter resins are dissolved from the husk more readily at higher pH values, since they are weakly acidic in nature. Color, likewise, is extracted better at higher pH values.

During mashing, the pH varies, but it may be in the neighborhood of 5.8 at the beginning of the process. Lager beer, when ready for cooling, may indicate a pH of about 5.2 to 5.5.

High-grade lactic, sulfuric, phosphoric, or other acids may be used to adjust the reaction of the medium. It is essential, however, to use an acid that will have no deleterious effect on the flavor of the beer or its value to the consumer.

*Concentration of Mash.* Since mashing is an enzymic process, the concentration of the mash influences the quality and quantity of the extract (refer to Fig. 28).

*Time.* In order to control the composition of the final mash, it is ortant to regulate the time permitted for mashing. The time allowed l be intimately connected with the other factors of enzyme reaction—perature, pH, concentration of substrate, and menstruum.

uring the last part of mashing especially, the grains and coagulated teins will settle to the bottom of the mashing tub. Although some nufacturers permit settling to take place in this tub and filter the wort allowing it to flow down through the layer of grains and coagulated teins, the wort passing out through a false bottom, others convey the terial from the mash tub to a lauter tub. The lauter tub is a special ering tank with a false bottom. Those which are wide and shallow e the quickest and best results. The filtered wort should be clear.

The grains that remain after the wort has been drawn off are "spent." ey are extracted or leached several times with water at a temperature 75 to 80°C., preferably 75°C., the process being known as "sparging." e sparged grain should finally contain less than 1 per cent of residual uble extract. It is used as cattle food, either in wet form or after ing.

**Boiling the Mash.** Wort containing hops is boiled for several rea- s: to concentrate it, to sterilize it, to inactivate the enzymes, to extract uble substances from the hops, to precipitate coagulable proteins and er substances, and to slightly caramelize the sugar. The addition of spargings to the main wort dilutes it, making concentration desirable. e danger of the growth of undesirable microorganisms in the wort is sened by sterilizing it and handling it under aseptic conditions. Inacti- ion of the enzymes aids in maintaining a wort of fairly constant com- sition for fermentation.

The hops are added to the wort in portions, some early and some toward end of the boiling process.

he usual boiling time is 1.5 to 2.5 hr.

he substances extracted from hops include bitter acids and resins, ntial oil, and tannin. The bitter acids and resins contribute to the atefulness, colloidal stability, and head retention of the beer. The er acids are humulone, or α-bitter acid, and lupulone, or β-bitter acid, pectively. By oxidation and polymerization the acids may be con- ted to soft resins. Both the acids and their corresponding resins pos- antiseptic properties and impart characteristic flavors to beer. mulone, however, possesses the strongest bitter flavor and the greatest iseptic action. A third resin, of little value to the brewing industry, is hard γ-resin.

A large proportion of the antiseptic action of hops is lost in the drying he hops, in their storage before use, in their boiling, and in the cooling, mentation, and storage of the beer in barrels.

ome flavor is imparted to beer from the essential oil of hops. Some he essential oil is volatile in steam, and most of it is lost during the ing of the wort, unless the hops are added toward the end of the boil- process.

annins are extracted principally from the hops during boiling, but e are extracted from the malt. The tannins from barley possess an unpleasant taste. Hence their removal by reaction with the protein the wort, before the hops are added, is advantageous.

Tannin aids in the precipitation of some of the nitrogenous substa of the wort. Some of the tannin complexes precipitate out during boil but others tend to become insoluble in the cold, giving rise to a cold h unless removed during subsequent cooling.

Tannins are negatively charged. They react readily with positi charged proteins, forming complexes that become less soluble as the t perature decreases. Tannins react less readily with electrically neu proteins, i.e., proteins that are unstable.

Boiling converts some of the tannin of the hops into phlobaph Protein phlobaphene forms also during the boiling of the wort, and, be insoluble in the hot wort, it precipitates out. Oxygen accelerates prot phlobaphene formation (Hopkins, 1938).

Agitation and circulation of the wort during boiling increases amount of precipitation and hence the quantity of sludge formed. Dur cooling, agitation is also of much advantage in increasing the amount precipitation. The greater the quantity of unstable compounds remo during boiling and cooling, the less likelihood there is of the formation precipitates in the finished product.

After the wort has been boiled, it is filtered through a hop straine remove the hops and precipitated proteins. The wort may, or may r be passed into a tank located above the cooler where settling may permitted for 0.5 to 1 hr. Some cooling takes place in this tank. T wort is then cooled by passing it over or through coolers, preferably w considerable agitation, and then filtered.

During cooling, the wort becomes aerated and the rH [*] may incre from 12 or 13 to 16 or 17. Protein-tannin compounds precipitate owing to their insolubility at the lower temperatures. A secondary p cipitation is induced wherein proteins and hop resins are adsorbed the surfaces of the protein-tannin compounds.

The rate at which the wort is cooled between the temperatures 60 and 21.1°C. has a direct bearing on protein-tannin precipitat (Sippel, 1938), brighter worts being secured through quicker cooling ra

Preventing contamination of the wort during cooling is essential.

**Fermentation.** *Yeasts.* Strains of *Saccharomyces cerevisiae* are co monly used in the manufacture of beer. The selection of an appropri strain is a most important factor in determining the character of the b

Brewery yeasts may be classified as top-fermenting yeasts, which used in the manufacture of ale, and bottom-fermenting yeasts, wh are used in the production of lager and other beers.

*S. carlsbergensis* Hansen is a bottom yeast, which has been used in breweries of Copenhagen and elsewhere. The Saaz yeast and *S. monac* *sis* are typical bottom yeasts also.

---

[*] This is a term which represents the "negative logarithm of the hypothet hydrogen pressure in equilibrium with the oxidation-reduction system" being stud M. Stephenson, "Bacteria Metabolism," 2d ed., Longmans, Green & Co., I New York, 1939.

About 1 lb. of yeast (0.2 lb. dry weight) may be used to pitch, or inocu- e, 1 barrel of wort.

*Conditions of Fermentation.* The cooled wort is pumped, or allowed flow by gravity, into the fermentors. The latter may be constructed of ass-lined steel, stainless steel, aluminum, wood, or other material com- nation. Closed tanks are frequently desired for the beer fermentation d are used to collect the carbon dioxide evolved and used later at e time of packaging. The open tank is usually desired for the ale fer- entation, because it provides ready access for skimming the foam heads ich accumulate during the fermentation. The modern fermentor is pro- ded with cooling coils for maintaining a relatively constant temperature fermentation.

The temperature of fermentation depends on whether it is a top or ttom fermentation. The starting temperature for a top fermentation, ich is the one in which ale is made, is usually about 55 to 60°F.; that r the bottom fermentation, in which beer is made, is usually about to 46°F.

As the fermentation proceeds, various substances accumulate on the rface of the medium, including hop resins, yeast cells, and proteinaceous aterial. This scum may be removed after the fermentation starts to ede, in order to improve the quality of the beer.

Certain very definite changes take place in the wort during fermen- ion. At a particular point near the end of the fermentation the yeast cculates and commences to settle. This change is known as a "break" d depends in part upon the pH for initiation. (Not all yeasts break, wever.) A large proportion of the fermentable sugars are transformed ethyl alcohol, carbon dioxide, glycerol, and acetic acid. Higher alcohols l acids are produced from protein derivatives.

A top fermentation is complete in 5 to 7 days; a bottom fermentation, 7 to 12 days.

**Storage.** The product which comes from the fermentors, now known 'beer" or "ale," contains undesirable substances in suspension. Occa- nally the malt beverage may be clarified by the use of a centrifugal e of clarifier after a short rest period. Usually, however, it is stored a temperature of about 32°F. for several weeks in order to permit the table proteins, yeast, resin, and other undesirable materials to precipi- . During the storage period, the beer matures, esters are formed, and harshness of the green product disappears (Fig. 30 shows a storage lar).

The malt beverage is usually chillproofed by the use of special enzymes. is procedure removes those proteins which would tend to precipitate at the lower temperatures and cause a haze.

**Finishing.** The matured beer is carbonated under pressure, using bon dioxide that is at least 99.5 per cent pure, until a final carbon xide content of 0.45 to 0.52 per cent is obtained. Gas is absorbed by beer, and oxygen is displaced. Exclusion of air in the finished beer is y important. The greater the amount of air displaced from the r, the better. The dissolved gas adds to the quality of the beer, aids

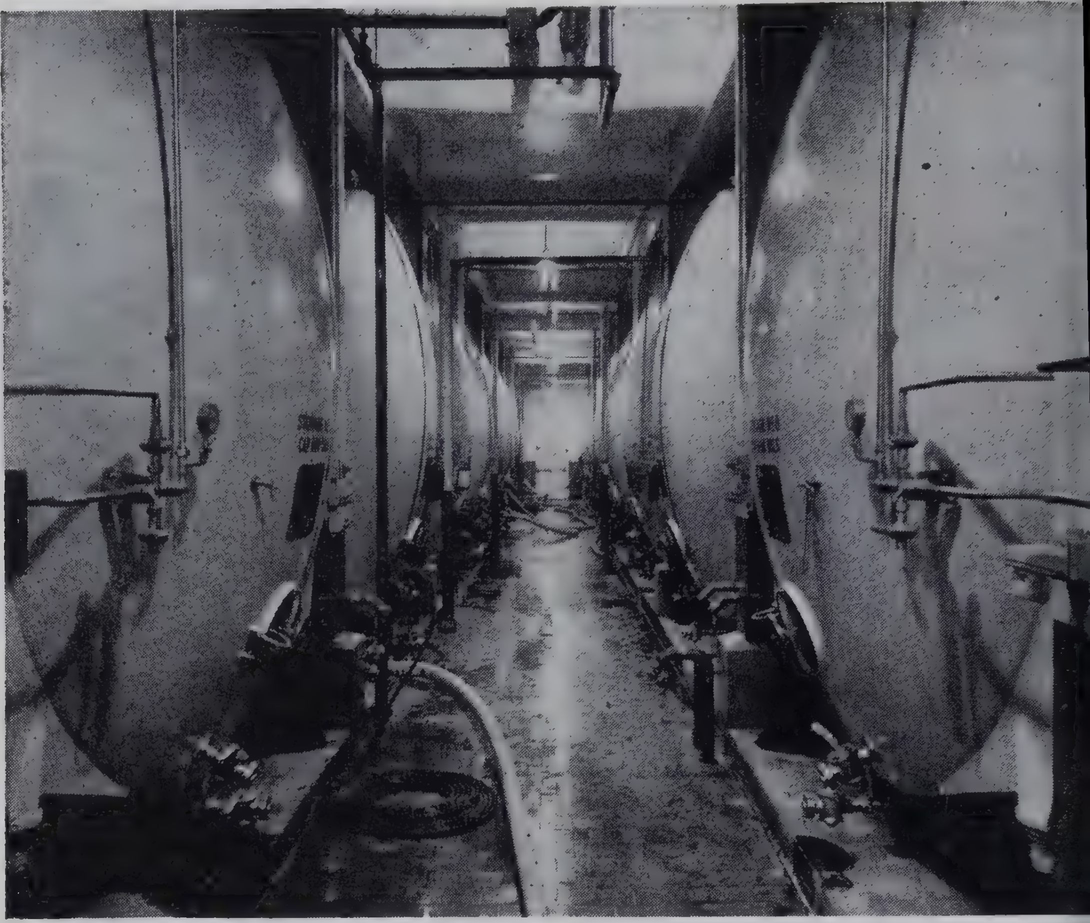


FIG. 30. Scene in modern beer storage cellar, showing glass-lined tanks where b is aged. (*Courtesy of E. J. McCann, Carling Brewing Company, Natick, Mass.*)

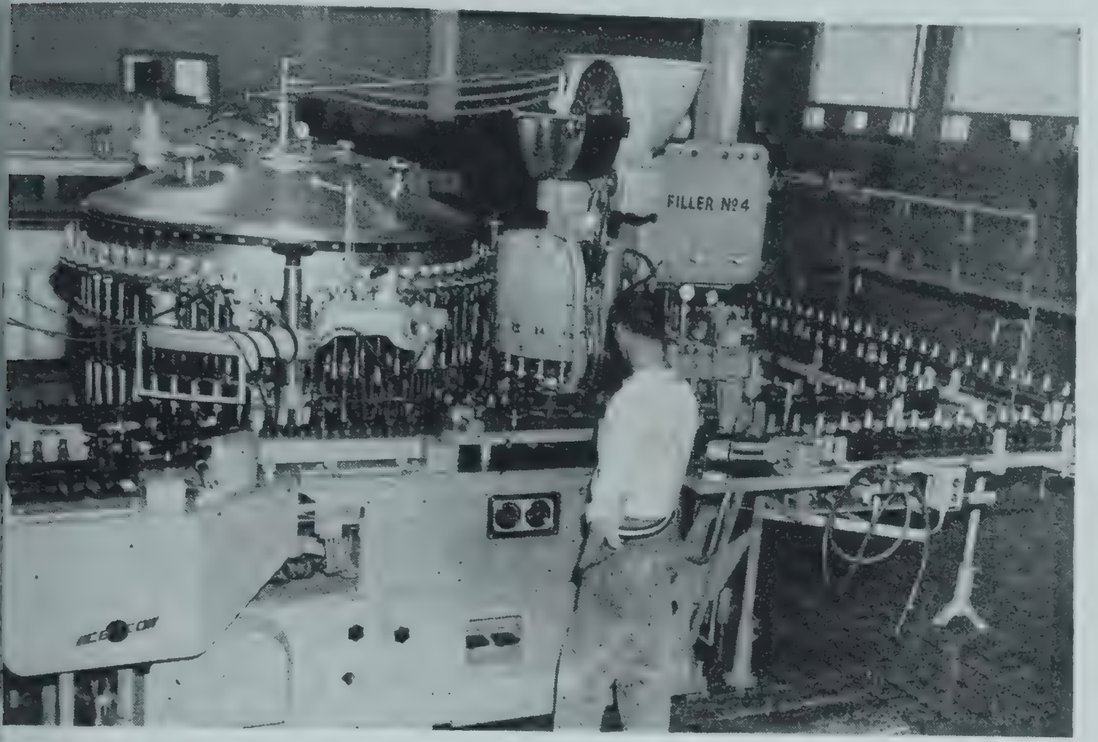
in the production and retention of foam, and helps to preserve t beer.

An alternate process for carbonating beer is known as the "krauseni process." The beer after storage is placed in pressure tanks or containe and approximately 15 per cent of beer in an initial active stage of f mentation (krausen) is added and mixed with it. The carbon dioxi evolved from the fermenting young beer impregnates the entire tank beer. Valves or bungs, attached to the pressure tanks, enable one regulate the final content of carbon dioxide in the beer (0.45 to 0.52 p cent). The krausening process usually requires 3 or 4 weeks. Afterwa the beer is stored for an additional period of 3 to 8 weeks. The acti of the yeast stops after the sugars have been consumed. Clarificati then proceeds.

The carbonated beer is filtered with diatomaceous earth.

The malt beverage is pumped through flow meters to measure the v ume for tax purposes and to Government cellars, where it may remain f 1 or more days before being placed in the final container.

The beverage may be filled into barrels of stainless steel, aluminum, wood (which usually contain 15.5 or 7.75 gal.); new or used bottles; cans (Mutschler, 1950). The product which goes into barrels is not pa teurized and thus has a relatively short storage life. Bottles are washe tomatically filled (Fig. 31), capped, rinsed with potable water, and nveyed to the pasteurizer (Fig. 32), where the contents are heated to a nperature of 140°F., or above, for a period adequate to destroy spoilage pes of microorganisms. A period of 55 min. may elapse between the



:. 31. High-speed bottle filler. (*Courtesy of E. J. McCann, Carling Brewing Com- ny, Natick, Mass.*)

e a bottle enters the pasteurizer and the time it emerges. Bottles are pected for defects, sometimes by electronic devices, labeled, and packed hipping cases. //

**Quality Control.** The modern brewer carefully controls the quality of product at all stages from the raw material to the finished product by d laboratory supervision.

**Defects in Beer.** *Turbidities* in beer may be caused by a number of tors: unstable protein, protein-tannin complexes, starch, resin, and roorganisms.

Gluten, or albumin, turbidity occurs at a low temperature generally. s type of turbidity is likely to appear when the malt has been improp- y dried in the kiln or when a barley with very high protein content is d. Warming the beer causes the turbidity to disappear.

Oxidation turbidity or haze is due in part to protein-tannin compounds. presence of oxygen; the shaking of the beer during its transportation; collisions of beer bottles, which impart supersonic and high-pitched nd vibrations; and sunlight affect the formation of oxidation haze. uration of the beer with carbon dioxide does much to prevent this bidity.

Tannin-protein hazes appear also at low temperatures. In order to duce stable, chillproof beers, which will not become hazy or turbid

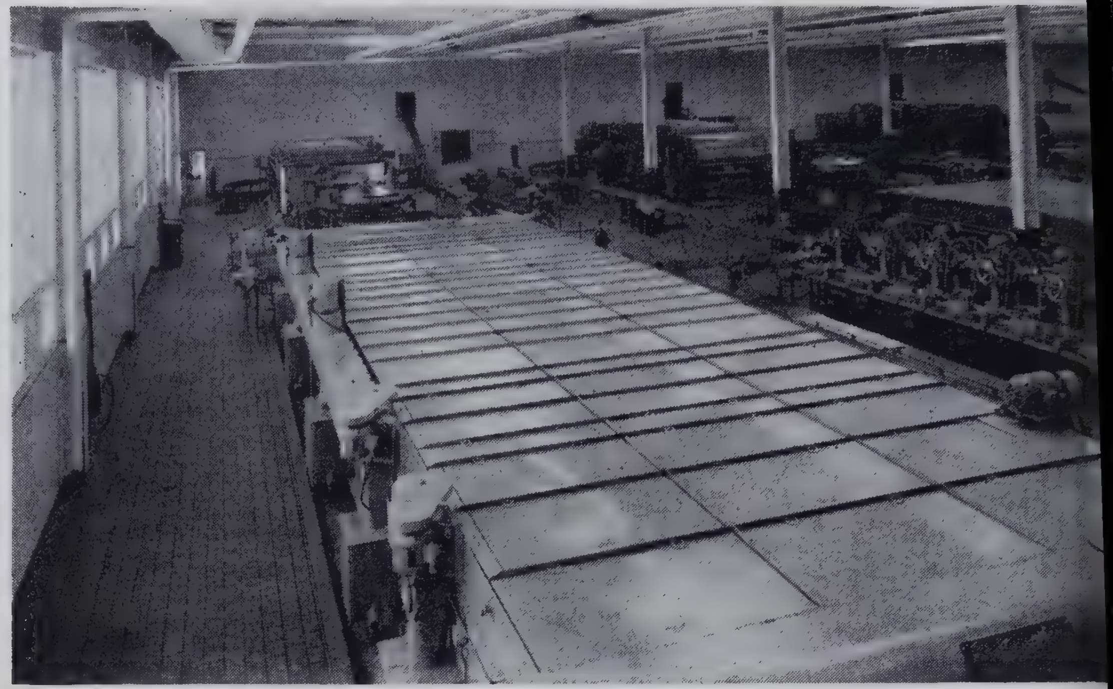


FIG. 32. Scene in bottle house of modern brewery. In the immediate foreground a pasteurizers, and behind these are bottle fillers and bottle washers. (*Courtesy of E. McCann, Carling Brewing Company, Natick, Mass.*)

when cold, the use of a small amount of a proteolytic enzyme preparatio is advantageous. The enzymes are usually added after the fermentatio although they may be added earlier. The enzymes in an acid mediu such as is found in a beer, render the beer stable and chillproof. Cred is due to Wallerstein (1911) for this discovery.

Starch turbidities develop as a result of the improper conversion starch during mashing. Lack of proper digestion at this time may be du to the use of a malt in which the diastase has been destroyed during th kilning. Sparging with water at a temperature much higher than 80° may also result in the production of turbidity. Amylases may be added the storage vats to remove starch turbidity.

The presence of resin oil containing pitch may, rarely, cause turbidit as may calcium oxalate. Proper filtration will prevent both types turbidities.

Yeast turbidity may be due to lack of proper clarification during th secondary fermentation, which in turn is caused by an unsatisfactor wort. The use of chips, or krausening (the addition of beer in an activ state of fermentation) is usually effective in correcting this type turbidity. Wild yeasts, especially of the *S. pastorianus* III species, pr duce turbidity. By excluding air and keeping the concentration of fe mentable sugars in the beer low, growth of yeasts is inhibited. A low r 12 or below, will also inhibit the growth of yeasts according to De Cler (1934). The use of pure cultures and proper sanitation of the plant shoul prevent the access and development of wild yeasts.

Among the turbidities caused by bacteria, those produced by sarcina are most common, especially in bottom fermentations. Bacterial tu ities are less common than turbidities caused by yeasts and frequently l contain yeasts as well as bacteria. Aseptic technique, the use of pure tures of yeasts, sanitation of the brewery, and a high content of hop iseptics should prevent bacterial turbidities.

*Faulty beer* may be the result of the use of a low-grade raw material he mash; the use of hops of poor quality or too much hops or prolonged ling of the hops; contact of the beer with iron, causing an inky taste; tact with tin; an unsuitable brewing water; young or green beer; bon dioxide deficiency; etc.

**Beer Infection.** The term "beer infection" or "beer disease" is com- nly used in describing the undesirable condition that occasionally sts in beers or ales as the result of the presence of microorganisms, ef among which are the bacteria. The microbial agents causing the nptoms may be designated as beer-infection microorganisms or beer- ease microorganisms.

The infection bacteria described in literature are included in five nilies: the Pseudomonadaceae, Bacillaceae, Achromobacteraceae, Mi- coccaceae, and Lactobacillaceae. However, the bacteria of only three these families, Pseudomonadaceae, Achromobacteraceae, and Lacto- illaceae, are of particular significance to the brewing industry. The ual number of genera involved is also small, as illustrated by Tosic, o reviewed the subject of beer-infection organisms in 1945 and reported t beer infections are caused by species of the following genera: *Aceto- ter*, *Lactobacillus*, *Streptococcus*, *Flavobacterium*, and *Achromobacter*. ere appears to be some doubt whether bacteria of the genera *Bacillus* l *Micrococcus* cause infections of malt beverages. The mere fact that reformers exist in the raw materials used in brewing is no indication t they are producers of infection.

t this point it may be well to consider some of the conditions encoun- d in the brewing operation that have in general pronounced effects on teria and that, in fact, may determine whether or not an organism will ive or develop in the beer. These conditions are as follows:

. The constituents of the wort are usually boiled for about 2.5 hr.
. The yeast fermentations are carried out at low temperatures.
. The pH values of the wort and beer are low.
. Hop antiseptics are present in the wort and beer.
. The beer is usually pasteurized.
. Anaerobic conditions usually prevail during fermentation and in beer.
. Ethyl alcohol is present in the fermentation mash and in the beer.

t is entirely conceivable that an unusually heat-resistant sporeformer, example, strain of one of the species of the genus *Bacillus* or *Clos- ium*, might survive the long boiling period of 2 to 2.5 hr. to which the t is subjected.

However, such bacteria are commonly Gram-positive and sensitive to antiseptics. A surviving sporeformer in order to cause infection ld have to be tolerant of a relatively low pH, of anaerobic conditions, of the alcohol normally found in a beer or ale. Thus, there are ample reasons why sporeformers may not be associated with beer infe tions.

Bacteria of the species of the *Acetobacter, Achromobacter, Flavobact rium, Lactobacillus,* and *Streptococcus* are destroyed within a relative short time at temperatures well below the boiling point. Hence, if beve ages contain these organisms, they have generally become infected durin one or more of the operations following the boiling of the wort.

The species of the genus *Acetobacter* are rod-shaped, though involutio forms may occur, Gram-negative, catalase-positive (with the exceptio of *A. peroxidans*), and aerobic and grow usually in the range of 5 to to 35 to 40°C. These bacteria produce acetic acid aerobically from eth alcohol and are tolerant of acid and of hop antiseptics in the amount commonly found in beers. They may be responsible for the production acidity or sourness in beers caused by acetic acid. Two other specie *A. capsulatum* and *A. viscosum,* may produce ropiness in beer, while third species, *A. turbidans,* may give rise to turbidity and sournes (Cosbie et al., 1943, 1944). The latter organism apparently has the abilit to develop when only a very small amount of oxygen is present.

The acetic acid bacteria, with the exception noted above, are dependent in their action upon the presence of oxygen. Consequently infections by species of the *Acetobacter* are limited to those cases where the fermented wort or beer is exposed to oxygen, as, for example, in cooks stored for a prolonged period, in empty beer barrels, and in the pitching yeast. Control is exercised by excluding oxygen from the fermented product and in using pure cultures of yeasts.

Two genera of the family Lactobacillaceae, namely *Lactobacillus* and *Streptococcus,* contain species which may be causes of serious trouble. Bacteria of the genus *Lactobacillus* are rod-shaped, Gram-positive, catalase-negative, microaerophilic, and tolerant to acid. They may be tolerant to hop antiseptics or often adapt themselves to its presence. *L. pastorianus,* synonyms of which are *Bacille des Bières Tournées* Pasteur and *Saccharobacillus pastorianus* Van Laer, gives rise to sourness in beers and a silky type of turbidity. It may produce lactic, acetic, and formic acids, alcohol, and carbon dioxide from fermentable sugars and grow in the range of 11 to 37°C.

According to Shimwell, *L. pastorianus* is probably the most common defect-producing organism to be found in top-fermentation breweries. Although difficult to remove entirely from a brewery, once it has gained entrance, the problem may be attacked in several ways. The plant should be kept clean, the yeast used for pitching should be pure, the residual fermentable extract should be kept at a minimum, the pH should be as low as possible consistent with the production of a quality product, the hop rate should be high at the time of racking, and finally the beer should be pasteurized, because *L. pastorianus* is destroyed by effective heat-treatment.

The streptococci causing trouble in beer are associated with such defects as "sarcina sickness," sourness, turbidity, and ropiness. Sarcina sickness is a disease of beer characterized by a honey-like odor. This par-ular odor is due to the production of diacetyl by the bacteria, which mbined with the normal beer odor, produces the honey-like aroma.

The species of bacteria concerned in the production of sarcina sickness, rbidity, sourness, and ropiness is *Streptococcus damnosus* (Claussen) imwell and Kirkpatrick. A synonym of this organism is *Pediococcus mnosus* Claussen. Shimwell also lists as possible synonyms *P. perniciius* Claussen, *P. cerevisiae* Balcke, and *P. sarcinaeformis* Reichard.

*Strept. damnosus* is nonsporeforming, Gram-positive, catalase-negative, aerobic, tolerant to acid but with an optimum pH of 5 to 6.

Two varieties of *Strept. damnosus* have been described. These are *ept. damnosus* var. *viscosus* and *Strept. damnosus* var. *pentosaceus* alters). The first of these varieties was isolated from Scotch ale by aussen and is characterized by the production of slime in beers that are urated with carbon dioxide. In flat beers, sarcina sickness is produced her than slime.

*Strept. damnosus* var. *pentosaceus* (originally named *Strept. tetragenus* r. *pentosaceus*) was isolated from Australian beer by Walters.

The family Bacteriaceae [1] contains two genera which are of considerable portance to the brewing microbiologists: *Achromobacter* and *Flavoterium*. *A. anaerobium* produces turbidity and off-odors in beers. *proteus* is an infection of brewers' yeast.

*A. anaerobium*, a Gram-negative, nonsporeforming anaerobe, is toler- of acids and hops. Actually it will grow at a pH of less than 4 and is inhibited by the action of hop antiseptics in the concentrations used breweries. It grows only in "primed" beers, but rapidly when the temature is elevated. Glucose and fructose, but not maltose, are utilized. ilky turbidity and odors of hydrogen sulfide (slight) and of apples are duced. Ethyl alcohol and carbon dioxide are formed. The organism estroyed by heat-treatment for 5 min. or longer at 60°C.

he infection of brewers' yeast, caused by *F. proteus*, has been debed by Shimwell (1936), and Shimwell and Grimes (1936). Shimwell ated the organism from "pitching" yeast. It is a Gram-negative, nonreforming, facultative anaerobe, which produces ethyl alcohol and acid gives rise to a parsnip-like odor and flavor in both hopped and opped wort. Its activities, however, are restricted to the primary fertation on account of its inability to grow at a pH of about 4.2. Since pH of the wort may be 5.2 or more at the beginning of the fermenta-., it may grow until the lowered pH inhibits further development.

able 48 summarizes some information concerning the defects of beer sed by bacteria.

**efinitions.** *Lager beer* is literally stored beer. The term "lager" is ived from *lagern*, a German verb meaning "to store." According to this nition, all beer would be lager beer. Lager beer is produced by bottom ientation and is rather high in alcohol and extract with a relatively proportion of hops.

*ock beer* is a heavy beer, dark in color and high in alcohol. It is ved for consumption in the early spring.

---

[1] Now Achromobacteraceae.

TABLE 48. A SUMMARY OF SOME INFORMATION CONCERNING THE DEFECTS OF BEER CAUSED BY BACTERIA *

| Defect | Specific causative agent | Morphology and size,† microns | Gram reaction | Catalase reaction | Relation to oxygen | Reaction to acid | Reaction to hops | Relation to temperature | Products |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Family Pseudomonadaceae | | | | | |
| Sourness | *Acetobacter aceti* | 0.4–0.8 by 1.0–2.0 | Neg. | Pos. | Aerobic | Tolerant | Tolerant | .............. | Acetic acid |
| | *A. acetosum* | 0.4–0.8 by 1.0 | Neg. | Pos. | Aerobic | Tolerant | Tolerant | Range, 8–36°C. | Acetic acid |
| | *A. industrium* | 0.3–0.8 by 2.4–20 | Neg. | Pos. | Aerobic | Tolerant | Tolerant | Range, 8–35°C. | Acetic acid |
| | *A. kützingianum* | Short, thick rods | Neg. | Pos. | Aerobic | Tolerant | Tolerant | Range, 7–42°C. | Acetic acid |
| | *A. lindneri* | Rods | Neg. | Pos. | Aerobic | Tolerant | Tolerant | .............. | Acetic acid |
| | *A. mobile* | Rods | Neg. | Pos. | Aerobic | Tolerant | Tolerant | .............. | Acetic acid |
| | *A. oxydans* | 0.8–1.2 by 2.4–2.7 | Neg. | Pos. | Aerobic | Tolerant | Tolerant | .............. | Acetic acid |
| | *A. pasteurianum* | 0.4–0.8 by 1.0 | Neg. | Pos. | Aerobic | Tolerant | Tolerant | Range, 5–40°C. | Acetic acid |
| | *A. suboxydans* | Short rods | Neg. | Pos. | Aerobic | Tolerant | Tolerant | .............. | Acetic acid |
| | *A. xylinum* | Short rods | Neg. | Pos. | Aerobic | Tolerant | Tolerant | .............. | Acetic acid |
| Ropiness | *A. capsulatum* | Coccoid rods 0.6–1.5 | Neg. | Pos. | Aerobic | Tolerant | Tolerant | .............. | Acetic acid |
| | *A. viscosum* | .................... | Neg. | Pos. | Aerobic | Tolerant | Tolerant | .............. | Acetic acid |
| Sourness and turbidity | *A. turbidans* | Short rods at 25°C., involution forms at 40°C. | Neg. | Pos. | Aerobic to micro-aero-philic | Tolerant | Tolerant | Grows in beers at 13°C., destroyed in 10 min. at 52°C. | Acetic acid |
| | | | | Family Achromobacteraceae | | | | | |
| Turbidity, off-odors, and off-flavors | *Achromobacter anaerobium* | Rods 1–1.5 by 2–10 | Neg. | .... | Anaerobic | Tolerant | Tolerant | Destroyed in 5 min. or more at 60°C. | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| yeast | *proteus* | Highly pleomorphic | | | anaerobe | at pH 4<br>Opt. pH = 5 | | at 18°C., destroyed in 5 min. at 54°C. | |
| Family Lactobacillaceae | | | | | | | | | |
| Sourness, silky turbidity | *Lactobacillus pastorianus* | 0.5–1.0 by 7–35 | Pos. | Neg. | Microaerophilic | Tolerant | Tolerant | Range, 11–37°C. | Lactic, acetic, and formic acid; alcohol; $CO_2$ |
| Sarcina sickness, sourness, turbidity, ropiness | *Streptococcus damnosus* | Cocci in pairs, tetrads, chains, clumps. About 1 | Pos. | Neg. | Anaerobic | May reduce pH to 3.8 | Sensitive but may acquire resistance | Opt. 21–25°C. | Lactic acid, diacetyl |
| | *Strept. damnosus* var. *viscosus* | Cocci in pairs, tetrads, chains | Pos. | Neg. | Anaerobic | May reduce pH to below 4 | Sensitive but may acquire resistance | ............. | Diacetyl, acid, etc. |
| | *Strept. damnosus* var. *pentosaceus* | Cocci in pairs, tetrads, chains | Pos. | Neg. | Anaerobic | Grows at pH 3.5 | Sensitive but may acquire resistance | Grows at 15°C. | Diacetyl, acid, etc. |

---

* Information obtained from articles by Tosic, Shimwell, and others, which are referred to in the references at the end of this chapter.

† Data secured from Bergey's "Manual of Determinative Bacteriology" 6th ed., The Williams & Wilkins Company, Baltimore, 1948) and other sources.

*Ale* is produced by top fermentation, is pale in color, tart in taste, high in alcohol and contains more hops than does beer.

*Porter* is a dark ale, high in extract and sweeter than the usual al taste. It is brewed from dark or black malt (malt roasted at a high t perature) to produce a wort of high extract. The flavor of hops is distinct than that of normal ale.

*Stout* is a strong porter that is high in alcohol and extract. It is d in color and possesses a sweet taste and strong flavor of malt. The flavor is more pronounced than that of porter.

*Weiss bier,* a beer made mainly from wheat, is produced by top mentation. It is rather light, possesses a marked flavor of malt and h is tart, and contains a large quantity of natural fermentation gas. I likely to be turbid in appearance.

*Cereal beverage* is beer containing less than 0.5 per cent alcohol. I sometimes known as "near beer."

**Brewery Thermometers.** The brewer frequently uses the Reaum thermometer instead of the Fahrenheit or Celsius (centigrade) scales. comparison of a few points on the three scales is given in the followi table:

Table 49. A Comparison of Some Points on the Reaumur, Centigrade, and Fahrenheit Scales

| Reaumur | Centigrade | Fahrenheit |
|---|---|---|
| −32 | −40 | −40 |
| −16 | −20 | − 4 |
| 0 | 0 | 32 |
| 4 | 5 | 41 |
| 8 | 10 | 50 |
| 16 | 20 | 68 |
| 32 | 40 | 104 |
| 48 | 60 | 140 |
| 52 | 65 | 149 |
| 56 | 70 | 158 |
| 80 | 100 | 212 |

It will be noticed that the Reaumur degree is equivalent to 1.25 cent grade degrees.

**Some Other Alcoholic Beverages.** Alcoholic beverages are consume in every country of the world. In some countries the use of a particul beverage has been passed down from antiquity, for example, kvass Russia, pulque in Mexico, taette in Scandinavia, and sorgho in Ma churia. A brief description of a few alcoholic beverages follows:

*Kvass* may be prepared by mixing equal parts of barley malt, r malt, and rye flour, adding boiling water, stirring, permitting the mash stand for several hours, adding more boiling water, and then inoculati with yeast and permitting fermentation to take place. Peppermint added to the fermented product for flavoring. This beverage is ve mon in Russia. Neighboring countries prepare similar drinks in which er starchy or saccharine materials may be used.

*ulque* is prepared by allowing the sweet juice of agave (the century nt is a common species) to undergo a spontaneous fermentation, which usually complete in about 1 day. Yeasts produce alcohol from the ars. Bacteria are present, which cause a rapid spoilage of the beverage ss it is consumed shortly after its manufacture. Pulque resembles r and has a flavor somewhat similar to that of sour milk. It is a very mon beverage in Mexico.

*aette* is an alcoholic beverage prepared from milk. Yeasts cause the racteristic changes in flavor. The product has a pleasing acid taste.

*orgho* is an alcoholic drink made from *Sorghum saccharatum*.

*ake* is the widely used alcoholic beverage of the Japanese. It is a ow rice wine containing 14 to 24 per cent of alcohol. There are vari- methods by which it is manufactured. Usually a *koji*, known as *e-koji* or *tane-koji*, is prepared. Steamed rice is inoculated with the res of *Aspergillus oryzae* and incubated at about 20°C. until the rice ell covered with mycelium. The *koji*, or starter, is mixed with steamed and some water and inoculated at a low temperature. Starch is con- ed to fermentable sugars. The thick liquid resultant from this enzyme rolysis and in which spontaneous fermentation usually takes place is wn as *moto*. *Koji*, *moto*, and more water are usually mixed. An alco- c fermentation ensues in which several yeasts may be active. *Sac- romyces sake, S. tokyo*, and *S. yeddo* are some of the yeasts charac- stic of sake.

*ombe* is an alcoholic beverage made by permitting millet seed to out and undergo conversion of the starch to sugars and by allowing a ntaneous fermentation of the saccharified starch in water.

*iti* is a wine made from the tubercles of *Osbeckia grandiflora*. It is a t African drink.

*inger beer* is characterized by its distinctly acid nature, the ginger or, and the presence of a small amount of alcohol. Carbon dioxide volved in considerable quantity. The raw materials are sugar (in o 20 per cent concentrations) and pieces of gingerroot. "Ginger-beer t" is added to a solution of the sugar. In the ginger-beer plant are a t, *S. pyriformis*, and a bacterium, *Bacterium vermiforme*. The yeast are entangled in the gelatinous sheaths of the bacteria. A symbiotic tionship apparently exists, for both the yeast and bacterium function when in each other's presence.

**egulations.** Regulations concerning malt beverages are published by U.S. Treasury Department, Internal Revenue Service, Alcohol and acco Tax Division.

**dditional Information.** Further details concerning brewing may be ined by reference to special sources, some of which are listed at the of this chapter. The books of Hopkins and Krause; Preece; Hind; el, Schwaiger, Leonhardt, and Merten; and Gray et al. ("Bottle Beer lity") will be found to be particularly informative. The research rts found in the *Proceedings of the Annual Meeting of the American*

*Society of Brewing Chemists* (published once a year) and emerging f the meetings of the European Brewery Convention Congress are of h caliber.

### Some American Brewing Periodicals

*American Brewer* (monthly), A. Schwarz Sons, New York, 1867–.

*American Brewers' Review* (monthly), American Brewers' Review Co., Chica 1887–1937.

*Brewers Bulletin* (semiweekly), The Brewers Bulletin, Inc., Chicago, 1907–.

*Brewers Digest* (formerly Siebel Technical Review) (monthly), Siebel Publishing Chicago, 1925–.

*Brewers Journal* (formerly *Western Brewer*) (monthly), H. S. Rich & Co., Chica 1876–.

*Brewery Age* (monthly), Brewery Age Publishing Co., Chicago, 1932–.

*Modern Brewer* (incorporated with *Brewer's Act*) (monthly), Modern Brewery, I New York, 1920–.

*The West Coast Brewer* (monthly), The West Coast Brewer, San Francisco, 193

Ulrich, C. F.: "Periodicals Directory, 1938," R. R. Bowker Company, New Yo 1938. (Contains a list of brewing periodicals.)

*Wallerstein Laboratories Communications* (quarterly), Wallerstein Laboratori New York.

### References on Brewing

Arnold, J. P., and F. Penman: "History of the Brewing Industry and Brewing Scien in America," G. L. Peterson, Chicago, 1933.

Atkin, L., and P. P. Gray: Influence of Wort Constituents on the Rate of Ferment tion, *Wallerstein Labs. Communs.*, **10**(31): 217–229 (1947).

Boruff, C. S.: Industrial Wastes, Recovery of Fermentation Residues as Feeds, *In Eng. Chem.*, **39**(5): 602 (1947).

Chrzaszcz, I., and J. Janicki: Recent Advances in the Fermentation Industrie *Chemistry & Industry*, **55**: 884 (1936).

Clark, W. M.: A Laboratory Mashing Apparatus, *Ind. Eng. Chem.* (*Anal. Ed.*), 11 41 (1939).

Cooper, I. M.: "References Ancient and Modern to the Literature on Beer and Ale United Brewers Industrial Foundation, New York, 1937.

Cosbie, A. J. C.: Brewing, the Story of a National Industry, *Wallerstein Labs. Com muns.*, **5**(15): 85–114 (1942).

———, J. Tosic, and T. K. Walker, *Jour. Inst. Brewing*, **49**: 88 (1943).

———, ———, and ———: *Jour. Inst. Brewing*, **47**: 382 (1944).

De Clerck, J.: Papers concerned with oxidation-reduction in relation to brewin *Wochschr. Brau.*, **51**: 196, 204, 213, 378 (1934); *Jour. Inst. Brewing*, **40**: 407, 41 (1934); *Am. Brewer*, **68** (January, 1935); *Modern Brewer*, **18**(6): 38 (1937 *Modern Brewer*, **19**(1): 40 (1938).

Ehrnst, L. E.: Uses Laboratory to Direct Process, Assures Quality, *Food Inds.*, **11**(2 76 (1939).

Gray, P. P., and I. Stone: Copper and Iron in Worts, Yeast and Beer, *Wallerste Labs. Communs.*, **5**(16): 193–199 (1942).

———, ———, and L. Atkin: Systematic Study of the Influence of Oxidation o Beer Flavor, *Wallerstein Labs. Communs.*, **10**(31): 183–194 (1947).

———, et al.: "Bottle Beer Quality: A 10-Year Research Record," Wallerstei Labs., New York, 1948.

Guilliermond, A.: "The Yeasts," translated and revised by F. W. Tanner, John Wile & Sons, Inc., New York, 1920.

Hanes, C. S.: Starch Structure and Enzyme Action, *New Phytologist*, **36**: 101, 18 (1937).

Helm, E., and B. Trolle: Experiments on Bottom Fermentation, I. Methodolog *Wallerstein Labs. Communs.*, **10**(30): 87–98 (1947).

Hind, H. L.: Pasteur to 1936: An Account of the Development of Science in Brewing *Chemistry & Industry*, **56**: 125 (1937).

——: "Brewing Science and Practice," Vol. I, "Brewing Materials," Vol. II, "Brewing Processes." John Wiley & Sons, Inc., New York, 1938 and 1940.
opkins, R. H.: Prevention Better Than Cure for Brewing Troubles, *Food Inds.*, **10**: 74 (1938).
——: Enzymes in Malting and Brewing, *Am. Brewer*, **76**(1): 34 (1943).
—— and C. B. Krause: "Biochemistry Applied to Malting and Brewing," 2d ed., D. Van Nostrand Company, Inc., Princeton, N.J., 1947.
rgensen, A.: "Practical Management of Pure Yeast," revised by Albert Hansen, Charles Griffin & Company, Ltd., London, 1936.
ch, J. R., O. Nelson, and L. Ehrnst: Viscometer for Routine Determination of Proteolytic Activity of Malts, *Ind. Eng. Chem.* (*Anal. Ed.*), **11**: 35 (1939).
ufer, S.: The Colorimetric Determination of Oxidation-reduction Potential and Its Application to Brewing Practice, *Am. Brewer*, December, 1935.
—— and R. Schwarz: "Yeast Fermentation and Pure Culture Systems," Schwarz Laboratories, Inc., New York, 1936.
ch, A. E., and A. L. Winton: "Food Inspection and Analysis," 4th ed., John Wiley & Sons, Inc., New York, 1920.
rs, H.: The Significance of Oxygen in Brewing, *Wochschr. Brau.*, **54**(49): 389 (1937).
—— and C. Enders: Acidity and Protein Turbidities in Beer, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **22**: 329 (1938).
es, R. H.: "Onderzoekingen over de Biersarcina," Drukkerij Gebroeders Hoitsema, Groningen, 1934.
ndlik, F.: Oxydations—Reduktionspotentiale im Bier, *Wochschr. Brau.*, **51**: 307 (1934); *Am. Brewer*, **68** (February, 1935).
tschler, W. J.: The Canning of Beer, *Wallerstein Labs. Communs.*, **13**(41): 115–120 (1950).
vak, C. A.: "Modern Brewing, A Practical Handbook of Contemporary Brewing Practice," 2d ed., Frank A. Roth Co., St. Louis, 1934.
—: "Twelve Treatises on Practical Brewing," Modern Brewer, New York, 1935.
ey, A. L.: "Brewing Formulas," Jersey Printing Co., Inc., Bayonne, N.J., 1937.
ons, W. M.: "Beer and Brewing in America. An Economic Study," United Brewers Industrial Foundation, New York, 1937.
en, M. A.: Enzymes in Brewing, *Ind. Eng. Chem.*, **26**: 1127 (1934).
ce, I. A.: "The Biochemistry of Brewing," Oliver & Boyd, Edinburgh, 1954.
r, E. J.: Yeast Propagation in the Modern Brewery, *Brewers Dig.*, **14**(2): 25 (1939).
, G. T.: How to Convert Brewery to Industrial Alcohol Productions, *Food Inds.*, 4(5): 46 (1942).
le, A. L.: Origin and Activity of Enzymes during Malting, *Wallerstein Labs. Communs.*, December, 1937, p. 13.
arz, R.: Brewing Processes, *Ind. Eng. Chem.*, **27**: 1031 (1935).
well, J. L.: *Jour. Inst. Brewing*, **42**: 119 (1936).
–: Some Facts and Fallacies in Brewing Bacteriology, *Jour. Inst. Brewing*, 4(12) (1938).
–: The Classification and Identification of Bacteria, with Special Reference to Beer Types, *Jour. Inst. Brewing*, **46**(6): 207 (1940).
–: The Lactic Acid Bacteria of Beer, *Wallerstein Labs. Communs.*, **4**(11): 41–48 1941).
–: The Gram Stain and Its Significance in Brewing Bacteriology, *Wallerstein abs. Communs.*, **8**(23): 23–33 (1945).
–: Yeast Contaminants, *Brewers Dig.*, **22**(4): 45T (1947).
- and M. J. Grimes: *Jour. Inst. Brewing*, **42**: 348 (1936).
, F. P., Jr.: Wartime Adjustments in Brewing Materials, *Modern Brewery Age*, (6): 29 (1943).
, G. B.: Recent Advances in Brewing Technology, *Food Research*, 3: 269 938).
E. H., Jr., F. H. Schwaiger, H. G. Leonhardt, and J. A. Merten: "The Pracal Brewer," Master Brewers Association of America, St. Louis, 1946.
stein, L.: U.S. Patents 995,820 and 995,824, 1911.

## CHAPTER 7

## WINE

Wine is the product made by the normal alcoholic fermentation of t juice of sound, ripe grapes, modified by the usual cellar treatment. Rel tively small, but significant, amounts of wine are made in the Unit States from raisins, apples, blackberries, peaches, cherries, loganberri oranges, currants, apples, boysenberries, apricots, grapefruit, pomegra ates, raspberries, elderberries, honey, rhubarb, pears, and strawberries. wine made from a fruit such as the foregoing is named after the fruit, f example, "peach wine."

**Wine Classes and Types.** A wine belongs to one of five classes: app tizer wines, red table wines, white table wines, dessert wines, or sparklin wines. The fourteen most popular types of wines in the United States a shown in Table 50, together with some similar types.

Special terms are used to describe wines. **Still wines** are those whic do not contain carbon dioxide and include the table, appetizer, and desse wines. **Sparkling wines** are those which are made effervescent by se ondary fermentation in the bottle or in bulk, or by carbonation. **Dr wines** are those which contain no sugar or relatively small amounts whic are difficult to detect by taste perception.

The alcohol contents of the five classes of wines are distinctive. Re and white table wines and sparkling wines usually contain 10 to 14 pe cent of alcohol by volume; appetizer and dessert wines, about 20 per cen

**Wine-making Areas.** A large part of the world's wine is made in th countries located near the Mediterranean Sea. France leads the world i wine making, followed by Italy, Spain, Algeria, Portugal, Rumani Argentina, Russia, Hungary, Yugoslavia, United States, Chile, Greec Bulgaria, the Union of South Africa, Germany, and other countries.

In the United States, California makes about 90 per cent of the win principally in the Sonoma-Mendocino, Napa Valley–Solano, Livermore Contra Costa, Santa Clara–San Benito–Santa Cruz, Lodi-Sacrament Escalon-Modesto, Fresno–San Joaquin Valley, and southern Californi districts. Other areas of wine making include the Finger Lakes an Hudson Valley districts of New York (the second most importa state); the Sandusky–Lake Erie and Cincinnati districts of Ohi the Puget Sound and Yakima Valley districts of Washington; th Willamette Valley of Oregon; Council Bluffs, Iowa; Missouri, particularl the river area; southwestern Michigan; the Egg Harbor district of Ne Jersey; the Charlottesville district of Virginia; the Aberdeen district o North Carolina; and the Ozark Mountains of Arkansas.

TABLE 50. CLASSES AND TYPES OF WINES

| Classes | Best-known types * |
|---|---|
| tizer wines | *Sherry* (dry to sweet) |
| | *Vermouth* (dry to sweet) |
| table wines | *Burgundy* (dry): |
| | Barbera |
| | Charbono |
| | Gamay |
| | Pinot noir |
| | Red Pinot |
| | *Claret* (dry): |
| | Cabernet |
| | Grignolino |
| | Zinfandel |
| | *Rosé* (dry to sweet) |
| | *Vino rosso* |
| e table wines | *Chablis* (dry): |
| | Chardonnay |
| | Pinot blanc |
| | Pinot |
| | Folle blanche |
| | *Rhine wine* (dry): |
| | Riesling |
| | Sylvaner |
| | Traminer |
| | Chianti (white) |
| | *Sauterne* (dry to sweet): |
| | Sauvignon blanc |
| | Semillon |
| | Chateau or Haut |
| rt wines | *Port* (red, white, or tawny) (sweet) |
| | *Muscatel* (sweet) |
| | *Tokay* (sweet) |
| ling wines | *Champagne* (pink or gold) (extradry to sweet) |
| | *Sparkling Burgundy* (semisweet to sweet) |

he 14 most popular types are shown in italics.

**ports.** In spite of the fact that hundreds of millions of gallons of
are made in this country annually, much wine is imported, particu-
from Italy, France, Spain, Germany, Portugal, Denmark and
oes, Chile, the Netherlands, and Greece. During the year 1956,
,590 gal. of wine were imported, which included 3,834,365 gal. of
wine, 970,555 gal. of dessert wine, 2,265,885 gal. of vermouth, and
85 gal. of sparkling wine, according to the Wine Advisory Board,
Francisco, Calif.

**oduction Statistics.** Data relating to the production of still and
ling wines by the leading states in the United States are presented
bles 51 and 52.

TABLE 51. STILL WINES: PRODUCTION AND TREATMENT OF WINES AFTER FERMENTA
BY LEADING STATES, FISCAL YEAR 1956 *

| State | Production of still wines,† wine gallons | Treatment after fermentatio (increase in volume) | |
|---|---|---|---|
| | | By sweetening | By addition wine spirit |
| California | 137,762,910 | 1,361,000 | 17,460,235 |
| New York | 7,786,400 | 546,794 | 200,131 |
| New Jersey | 4,014,498 | 147,628 | 263,396 |
| Illinois | 3,311,923 | 417,805 | |
| Michigan | 2,180.861 | 116,150 | 44.674 |
| Washington | 2,019,255 | 71,172 | 104,541 |
| Virginia | 706,298 | 33,831 | 45.107 |
| Ohio | 534,798 | 21,646 | 26,337 |
| Total | 159,146,862 | 2,759,769 | 18,193,847 |

* Data obtained from U.S. Department of the Treasury, Internal Revenue Ser Alcohol and Tobacco Tax Division, *Statistics Relating to the Alcohol and Tob Industries for the Fiscal Year Ended June* 30, 1956, *Publication* 67, 1956.

† Represents the amount removed from fermentors exclusive of substandard w produced as distilling material for the production of brandy.

‡ Brandy and spirits fruit withdrawn for addition to wine totaled 36,411,116 tax

TABLE 52. PRODUCTION OF SPARKLING WINE, JULY 1, 1955 TO JUNE 30, 1956 *

| State | Gross production, gal. † | No. of premises ‡ |
|---|---|---|
| California | 1,074,974 | 46 |
| New York | 907,140 | 24 |
| New Jersey | 119,220 | 8 |
| Ohio | 56,099 | 12 |
| Michigan | 12,036 | 5 |
| Missouri | 8,019 | 2 |
| Kentucky | 19 | 1 |
| Total, United States | 2,177,507 | 110 |

* Data obtained from U.S. Department of the Treasury, Internal Revenue Serv Alcohol and Tobacco Tax Division, *Statistics Relating to the Alcohol and Tobc Industries for the Fiscal Year Ended June* 30, 1956, *Publication* 67, 1956.

† Produced by secondary fermentation or carbonation. Includes 11,416 gal. carbonated wine.

‡ Operated during any part of the year.

**Wine Making.** Although the fundamentals of wine making are si lar in all the principal regions in the United States, methods vary to so extent in the different regions owing to the varieties of grapes used, climate, and other factors. For example, a lower temperature may used in the pasteurization of wines produced in the eastern portion the United States on account of the greater acidity of the wine.

view of the magnitude of the industry in California, special attention will be devoted to discussing wine making as it is practiced there, rating with a description of the production of red wine.

e reader desirous of securing additional knowledge of wine making find numerous references at the end of this chapter, which should e to be both interesting and instructive.

**ie Making of Red Wine.** *Outline of the Process.* Selected grapes ie proper maturity are crushed and stemmed; treated with sulfur de, or a sulfite, or pasteurized; and inoculated with a starter contain- ı pure culture of yeast. After a short fermentation period the wine awn off, placed in storage tanks for further fermentation, racked, d for aging, clarified, and packaged.

*tails of the Process.* 1. THE GRAPE. The production of a fine wine be regarded as commencing with the selection of the best variety of · for use in its manufacture. Amerine and Winkler (1944) recom- led the varieties shown in Table 53 for regions in California. The ive value of some white and red varieties of grapes for table and rt wines is illustrated in Table 54.

ie quality of the grapes of a given variety will depend upon the con- ns under which they are grown—soil, climate, and other condi- .

apes should be gathered at the proper stage of maturity. In order termine the degree of maturity, representative bunches of grapes icked, and the Balling degree of their juice is determined. A reading to 23° Balling is usually given by the juice of the grapes when they t the optimum stage of maturity.

HANDLING THE GRAPES. In gathering the grapes and transporting to the winery, the prime purpose should be to have them arrive in ery best condition possible. The grapes should be picked with care, d in clean containers, and protected from deterioration. Careful vision of the handling of grapes is essential.

CRUSHING THE GRAPES. Grapes are crushed and stemmed by machine. hemical composition of the metal used in the construction of this inery and other equipment about the winery is important. Iron and are used in some wineries but are undesirable for they may cause ing of the wine, forming so-called "ferric casse." The tin and copper lved from bronze by grape juice, if sufficient in quantity, may cause r and color losses during the aging process. Stainless steel, nickel, conel should be used in preference to iron, ordinary steel, and many zes.

the grapes are not picked when cool, it is desirable to permit them to overnight before they are crushed.

TREATMENT BEFORE FERMENTATION. Grapes contain on their surfaces ied flora of microorganisms—molds, yeasts, and bacteria. It is quite ble that the juice of crushed grapes will produce a good wine without pecial precautions, but a wine manufacturer cannot afford to gamble spect to the quality of his wine. He can do much to ensure the ty of his final product by destroying or inhibiting the development

Table 53. Recommended Varieties of Grapes for Regions in California *, †

| Region 1 (fewer than 2,500 degree-days) ‡ | Region 2 (2,501–3,000 degree-days) ‡ | Region 3 (3,001–3,500 degree-days) ‡ | Region 4 (3,501–4,000 degree-days) ‡ | Region 5 (4,001 or more degree-days) ‡ |
|---|---|---|---|---|
| | | White wines | | |
| High-quality dry table wines:<br>White Riesling<br>Chardonnay<br>Sauvignon blanc | High-quality dry table wines:<br>Pinot blanc<br>White Riesling<br>Sauvignon blanc<br>Standard, special, or blending dry table wines:<br>Semillon<br>Sylvaner<br>Folle blanche | Natural sweet wines:<br>Semillon<br>Sauvignon blanc<br>Muscat Canelli<br>Standard, special, or blending wines:<br>French Colombard<br>Pinot blanc | Standard-quality wines:<br>French Colombard<br>Muscatels:<br>Malvasia bianca<br>Muscat Canelli<br>Orange muscat<br>Dessert wines:<br>Mission<br>Palomino<br>Verdelho | Muscatels:<br>Malvasia bianca<br>Orange muscat<br>Dessert wines:<br>Palomino<br>Mission<br>Grenache |
| | | Red wines | | |
| High-quality table wines:<br>Cabernet Sauvignon<br>Pinot noir<br>Standard or blending wines:<br>Gamay<br>Grenache | High-quality table wines:<br>Cabernet Sauvignon<br>Standard, special, or blending wines:<br>Grenache<br>Petite Sirah<br>Refosco | Standard, special, or blending table wines:<br>Barbera<br>Carignane<br>Refosco<br>Dessert wine:<br>Trousseau | Standard table wine:<br>Barbera<br>Bulk-quality table wines:<br>Carignane<br>Refosco<br>Dessert wines:<br>Tinta Madeira<br>Trousseau<br>Muscatel:<br>Aleatico | Dessert wines:<br>Trousseau<br>Tinta Madeira<br>Special or blending wines:<br>Salvador |

E 54. Relative Value of White and Red Varieties of Grapes for Table and Dessert Wines *

| Table wines | | | Dessert wines | | |
|---|---|---|---|---|---|
| Varieties | Relative value | Productivity | Varieties | Relative value | Productivity |
| e: | | | White: | | |
| ardonnay | 92 | Low | Palomino | 82 | High |
| ite Riesling | 88 | Low | Muscat Canelli | 82 | Low medium |
| nillon | 85 | Medium | Orange muscat | 77 | High |
| vaner | 85 | Low medium | Gray Riesling | 76 | High |
| ot blanc | 83 | Low | Mulvasia bianca | 73 | Medium |
| | | | Red: | | |
| bernet Sauvignon. | 98 | Low | Tinta Madeira | 81 | High medium |
| ot noir | 92 | Low | Mission | 79 | High |
| nache | 82 | High | Trousseau | 77 | High |
| gnolino | 79 | Medium | Grenache | 77 | High |
| bera | 78 | High medium | Zinfandel | 73 | High medium |
| | | | Aleatico | 73 | Medium |

formation obtained from M. A. Amerine and A. J. Winkler, Composition and
ty of Musts and Wines of California Grapes, *Hilgardia*, 15(6): 493–673 (1944).
nformation on additional varieties of lower relative value refer to article by
ine and Winkler.

---

formation obtained from article by M. A. Amerine and A. J. Winkler, Composi-
nd Quality of Musts and Wines of California Grapes, *Hilgardia*, 15(6): 493–
944).

the term "degree-days" is meant the summation of heat above 50°F. for the
of April to October, inclusive. For example, if the mean temperature for
as 75°C., the summation would be (75 − 50)31 = 775 degree-days.

*gion* 1: Napa and Oakville in Napa County; Hollister and San Juan Bautista
Benito County; Woodside in San Mateo County; Mission San Jose in
da County; Saratoga in Santa Clara County; Bonny Doon and Vinehill districts
ta Cruz County; and Guerneville, Santa Rosa, and Sonoma in Sonoma County.

*on* 2: Soledad in Monterey County; Rutherford, St. Helena, and Spring
ain in Napa County; Santa Barbara in Santa Barbara County; Almaden
rd, Evergreen, Guadalupe district, and Los Gatos in Santa Clara County; and
Ellen in Sonoma County.

*ion* 3: Livermore and Pleasanton in Alameda County; Calpella, Ukiah, and
nd in Mendocino County; Calistoga in Napa County; Alpine in San Diego
y; Templeton in San Luis Obispo County; Loma Prieta in Santa Cruz
y; and Alexander Valley, Asti, and Cloverdale in Sonoma County.

*on* 4: Guasti in San Bernadino County; Escondido in San Diego County;
o, Escalon, Lockeford, Lodi, and Manteca in San Joaquin County; Cordelia in
County; Ceres, Hughson, and Vernalis in Stanislaus County; Ojai in Ventura
; and Davis in Yolo County.

*on* 5: Fresno and Sanger in Fresno County; Madera in Madera County;
and Livingston in Merced County; and Trocha in Tulare County.

ed from M. A. Amerine and A. J. Winkler, Composition and Quality of Musts
ines of California Grapes, *Hilgardia,* 15(6): 493–673 (1944).

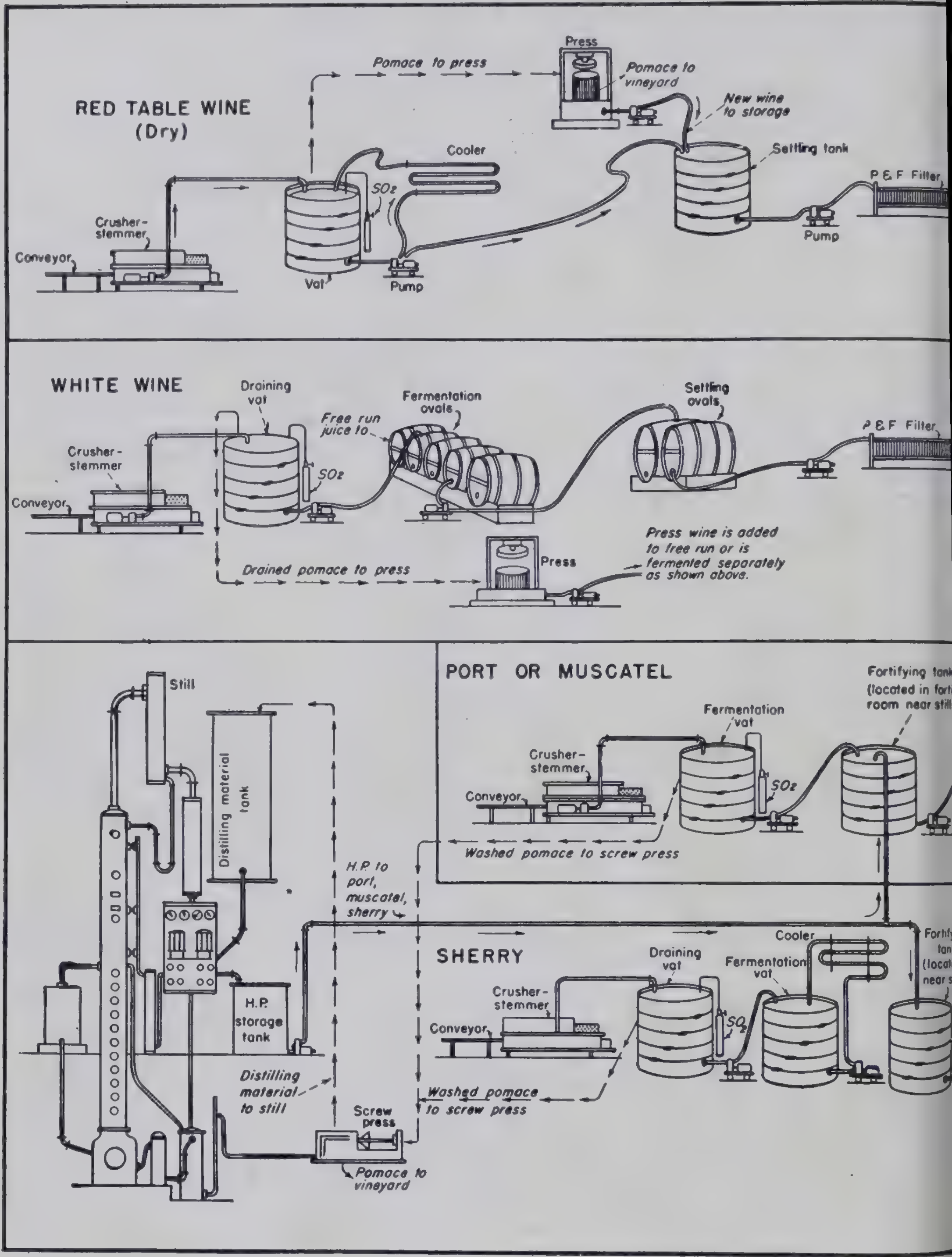
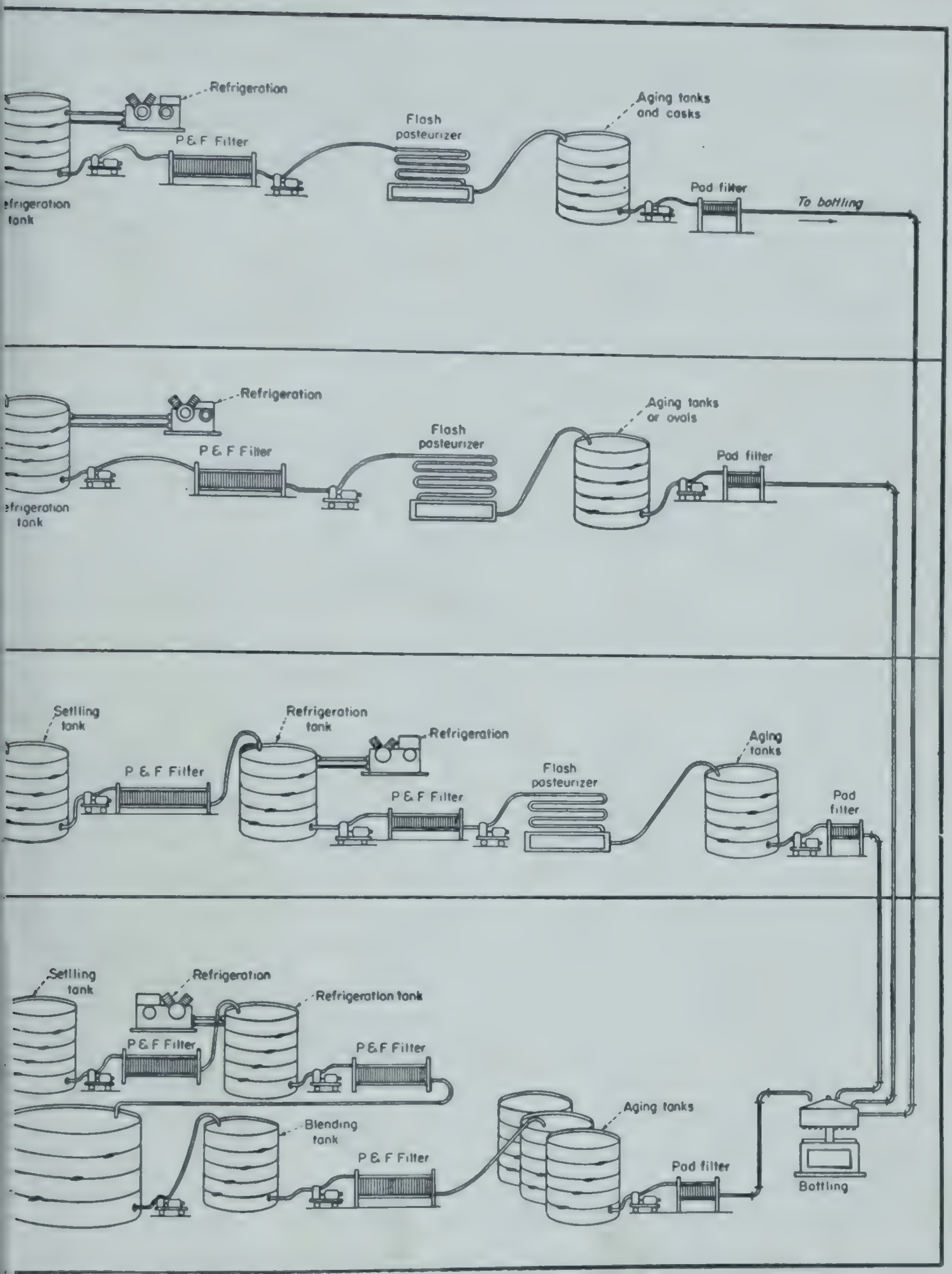

RED TABLE WINE
(Dry)
Conveyor
Crusher-stemmer
Vat
SO2
Cooler
Pump
Pomace to press
Press
Pomace to vineyard
New wine to storage
Settling tank
Pump
P & F Filter
WHITE WINE
Conveyor
Crusher-stemmer
Draining vat
SO2
Free run juice to
Fermentation ovals
Settling ovals
P & F Filter
Drained pomace to press
Press
Press wine is added to free run or is fermented separately as shown above.
Still
Distilling material tank
H.P. to port, muscatel, sherry
H.P. storage tank
Distilling material to still
Screw press
Pomace to vineyard
PORT OR MUSCATEL
Conveyor
Crusher-stemmer
Fermentation vat
SO2
Fortifying tank (located in forti room near still
Washed pomace to screw press
SHERRY
Conveyor
Crusher-stemmer
Draining vat
SO2
Fermentation vat
Cooler
Fortify tan (locat near s
Washed pomace to screw press

FIG. 33. Flow diagram of wine manufacture. [*Courtesy*

of the microorganisms found on the grapes and by the use of starters co
taining pure cultures of the specific yeast desired.

Sulfur dioxide or sulfites destroy or inhibit the growth of many und
sirable types of microorganisms—acetic acid bacteria, wild yeasts, a
molds—with a minimum amount of injury to the true wine yeast. Usual
2 to 6 oz., or twice the quantity of potassium metabisulfite, is added p
ton of crushed grapes, the quantity used depending on the condition of t
grapes—their maturity, the degree of contamination with molds, the te


Refrigeration
Aging tanks and casks
Flash posteurizer
P & F Filter
efrigeration tank
Pad filter
To bottling
Refrigeration
Aging tanks or ovals
Flash posteurizer
P & F Filter
Pad filter
efrigeration tank
Settling tank
Refrigeration tank
Refrigeration
Aging tanks
P & F Filter
Flash posteurizer
P & F Filter
Pad filter
Settling tank
Refrigeration
Refrigeration tank
P & F Filter
P & F Filter
Blending tank
Aging tanks
P & F Filter
Pad filter
Bottling

. Cruess and C. R. Havighorst, Food Inds., 20(4): 523 (1948).]

ure of the crushed product, and other factors. The largest quantities
sed when the grapes are overripe, moldy, or relatively warm.

teurization may be used in place of sulfites but is not usually con-
d to be so desirable.

HE FERMENTATION. The selection of a yeast, the nutrient sub-
s in the must (grape juice), the concentration of the sugar, the
y, the oxygen supply, and the temperature are factors that must be
vised in respect to fermentation.

*S. cerevisiae* var. *ellipsoideus* is the yeast used for the fermentatio must. Selected strains, such as the Burgundy or Tokay strains, are quently used. Many strains, bearing different names, are known.

A starter is prepared from a pure culture of the selected yeast. P teurized must is used as the culture medium in preparing the starter, magnitude of which should represent 2 to 5 per cent of the volume the crushed grapes being inoculated.

It is usually unnecessary to add any substances for the nutrition of yeast since the crushed grapes are an adequate source of supply. On r occasions ammonium sulfate or phosphates may be added.

Joslyn and Cruess state that the optimum concentration of sugar 22° Balling. The use of much higher concentrations of sugar favors production of more than 13 per cent of alcohol by volume. Since alco tends to inhibit the fermentation when present in concentrations of 13 15 per cent by volume, a maximum of 13 per cent is usually desirab The concentration that actually inhibits fermentation depends in part the temperature of fermentation, the tolerance of the yeast for alcol decreasing with increasing temperature. The approximate concentrati of the alcohol that will be produced in the wine can be predetermined multiplying the Balling reading of the must by 0.575.

It is permissible to reduce the concentration of sugar in must by t addition of water (Cruess, 1947). Another practice is to mix the jui with the high sugar concentration with a juice of low sugar concentratio Occasionally sugar may be added to must.

Grapes that have been permitted to become too mature are frequent of low acidity. Fruit acid—tartaric, citric, or malic acids—may be add to restore the normal acidity.

A large supply of oxygen is essential for the rapid multiplication yeast cells and the starting of the fermentation, as stated under yea manufacture (Chap. 3), while the later stage characterized by alcoh and carbon dioxide production rather than growth proceeds best und nearly anaerobic condition.

Approximately 6 hr. after the crushed grapes are treated with sulf dioxide or sulfite, the starter is added. Thereafter the contents of t tank are mixed, or stirred, at least twice a day, except during the ma fermentation, to facilitate aeration, temperature equalization, and t extraction of color and tannin. Normally a "cap" forms on the surfa of the fermentation vat, which contains grape skins, pieces of stem, seed and other suspended matter. To mix the contents of the tank, one ma punch down the cap or pump juice from the bottom of the vat over t surface of the must.

The amount of aeration produced by mixing the contents of the tan is determined by the effectiveness of the procedure and by the frequen at which the operation is repeated. Provided that the fermentation slow at the beginning, or near the end of the incubation period, the supp of oxygen may be increased by more frequent mixing of the contents the vat. However, the must should not be overaerated during ferment

n. for overaeration is likely to produce a wine of inferior quality, espe- lly in so far as color and flavor are concerned.

Fermentation should be carried out at carefully controlled tempera- es. The finest wines are produced usually at temperatures below 85°F. .4°C.). The development of bouquet and aroma are favored by main- ning the fermenting must at low temperatures, around 70 to 75°F. .1 to 23.9°C.), for example. A temperature range of 70 to 90°F. (21.1 32.2°C.) is satisfactory. When the temperature rises to 85°F. .4°C.) or, at the most, to 90°F. (32.2°C.), the mash should be artifi- ly cooled. Temperatures above 95°F. (35°C.) are considered unsafe, le the fermentation is inhibited usually at temperatures of 97 to °F. (36.1 to 37.8°C.). Fermentations cease at a temperature of 105°F. .5°C.) generally. Undesirable bacteria develop at the higher tempera- es. Accordingly the quality of the wine is impaired. Obviously, at too temperatures, the fermentation is too slow to be practical.

During the fermentation, records of the temperature and the Balling ree should be made at least twice a day, one set of observations being orded on the side of the fermentation vat in order that the progress he wine may be followed.

After 3 to 5 days of active fermentation, sufficient tannin and a maxi- m of color have been extracted from the skin of the grape. Extraction acilitated by the agitation of pomace (skin, seeds, and pieces of stems) ing fermentation, by the ethyl alcohol produced from the grape sugar, the heat of fermentation, and by the mechanical breaking up of skin.

The wine maker decides when the color and tannin content are satis- ory and then draws off the wine to separate it from the pomace. He s not wait for all the sugar to be fermented. At the time of drawing the wine, the Balling reading may be 0 to 4°. It is not considered isable to mix the wine drawn off ("free-run wine") with that expressed n the pomace, for the latter is of lower quality.

FURTHER FERMENTATION. The free-run wine is placed in closed age tanks, equipped with bungs that allow the excess carbon dioxide scape. An atmosphere of carbon dioxide over the wine tends to bit the development of acetic acid bacteria and other aerobic types nicroorganisms. The fermentable sugar is usually consumed in 7 to ays at a temperature of 70 to 85°F. (21.1 to 29.4°C.).

the after fermentation becomes sluggish before the sugar is utilized, yeast may be activated by pumping over the wine.

RACKING. By "racking" is meant the drawing off of the wine from lees or sediment. Potassium bitartrate ($KHC_4H_4O_6$), i.e., cream of ar, is found in the lees. This substance is less soluble in alcohol than ater and precipitates out more rapidly at low temperatures.

ine is racked to facilitate its clearing and to prevent undesirable ors from being extracted from the old yeast.

STORAGE AND AGING. Two important changes take place during age and aging: clearing of the wine and the development of flavor.

In a new wine there are present substances which, if not removed. produce a sediment and probably cloudiness. These substances incl tartrates, certain proteins, and other matter. Naturally these substa would be removed by racking and filtration during a somewhat l storage and aging process, but the modern trend is to hasten the rem of these substances by methods that involve flash pasteurization precipitate certain proteins), cooling to room temperature and the 24 to 27°F. (−4.44 to −2.78°C.), and holding at the latter tempera for a few days. Filtration is carried out in the cold. Since the acid tent of the wine is frequently reduced by the foregoing rapid pro it is customary to adjust the acidity with citric or tartaric acids, former acid being preferred. The wine is placed in tanks for aging.

Wine storage tanks are generally constructed of white oak or redw white oak being the better of the two. The tanks are completely fi with wine and sealed to prevent the access of large quantities of oxy which would favor the growth of acetic acid bacteria and *C. mycoder* (wine flowers). Some wine is always lost through evaporation. C sequently, the tanks should be inspected regularly and kept filled w wine. Periodically the wine is racked. During racking and filli especially, carbon dioxide is lost while some oxygen is absorbed. A sm amount of oxygen accumulates in the headspace over the wine.

Flavor, which is due to a combination of taste and odor, is develo in wine as a result of oxidative changes and ester formation.

Aging proceeds slowly until oxygen becomes available in small qua tities. It is inhibited by the presence of large quantities of carbon dioxi by sulfur dioxide, and by the exclusion of air. New wines stored in a tight bottles do not age properly.

Alcohols, aldehydes, tannins, and other substances present in the wi are oxidizable. Alcohols may be converted to aldehyde and subsequen to acids by oxidation. Aldehydes form acetals with alcohol.

Combinations of alcohols with acids give rise to esters, which are imp tant in the production of aroma or bouquet. Although opinions dif concerning the importance of esters, it is recognized that the nature volatile esters is of greater significance than the quantity. The esters acetic acid contribute much to the flavor and bouquet of wine.

The time required for aging varies with the type of wine and the co ditions. A dry wine may require at least 2 years. Some fine wines a aged for 5 or more years.

9. CLARIFICATION. Wines may clear naturally over a period of ti but resort is frequently made to the use of finings followed by filtrati heating, refrigeration, or combinations of the foregoing. Fining age which include such substances as casein, gelatin and tannin, bentonite ( Wyoming origin, if possible), isinglass (fish protein from the sturgeo white of egg, and Spanish clay, are mixed with the wine carefully acco ing to direction, or preferably after laboratory tests have been carr out with small portions of the wine and the fining agents. The improp use of some of the fining agents may, in itself, be a cause of clouding of t wine.

iltration is carried out with filter aids. For further details the reader ferred to "The Principles and Practice of Wine Making," by Cruess, to the more recent publications on this subject.

). PACKAGING. The clarified wine is placed in oak barrels for bulk and in bottles or in cans for unit sale.

ottles of small and medium size may be pasteurized for 30 min. 40°F.

**hampagne.** Specially selected white table wines are used in the ing of champagne. Sugar, usually invert, in a concentration of 4 to g. per liter of wine, is added for each atmosphere of pressure desired ie champagne. Since a final pressure of 6 atmospheres is often desired, means that around 26 g. of sugar may be added per liter, depending, ourse, upon the amount of residual sugar already present in the wine. ic acid to provide about 0.75 per cent total acid is added. Finally, it 3 per cent by volume of a champagne yeast starter is added, and he ingredients are thoroughly mixed together. The bottles (less than l. in size) are filled, while agitation is continued, and corked. The s are secured with a steel clamp ("agraffe"). Afterward the bottles stored horizontally at a temperature of approximately 54°F. (or er or lower). Low temperatures are favored because of improved na and flavor in the final product, more efficient precipitation of the m of tartar, inhibition of bacterial growth, and less breakage of les. Higher temperatures, up to 70°F., are used occasionally to accel- e the process.

ie normal time for the bottle fermentation is about 6 months. At the of this period, the bottles are removed from the stack, agitated, and aced. Aging of the wine takes place over the sediment for a period months to 4 years. Final sedimentation of the yeast cells and tar- s may be accelerated by reducing the temperature of the wine to t 25°F. and holding it at this level for 1 to 2 weeks.

arification of the champagne is brought about by working the sedi- down the side of the bottle and on to the cork. This is accomplished, a period of 2 to 6 weeks, by turning the bottles daily, clockwise and erclockwise, and gradually increasing the angle of the bottles (tilt- on the disgorging racks. The sediment and some of the wine in the of the bottle are frozen at a temperature of 5°F., after the entire nts have been cooled to below 50°F. The agraffe is then removed, he sediment is disgorged as a result of the pressure contained in the e. Immediately after this, a clear mixture of sugar, aged wine, and dy ("dosage") is added to replace the material lost during the dis- ng. Only a small quantity of sugar is added to the best champagnes, are the driest. The bottles are recorked and the agraffe re- d.

**lk Process.** A bulk process for making sparkling wines was devel- by Charmat in 1907. The tank used for the secondary fermentation ecially constructed to withstand high pressures and is lined on the or with stainless steel, glass, enamel, or other material. The modern are equipped with facilities for controlling the temperature by circulating brine or warm water through a jacket. Such tanks may veniently hold 500 to 1,000 gal. of wine (Hull et al., 1951).

As in the champagne process, invert sugar is added to a selected wine in sufficient amount to yield a pressure of 4 to 6 atmosph Champagne yeast, representing about 5 per cent of the volume o *tirage*, is added. The secondary fermentation proceeds at a tempera of 62 to 68°F. for about 30 days. The wine is cooled to about 25°F. allowed to remain at this temperature for up to 4 weeks to facilitate deposition of cream of tartar and yeast cells. It is filtered at isob pressure into a second container of similar construction, allowed to s for about a month, and bottled isobarically. The bottles are cor wired, and aged at 66 to 70°F. (Hull et al., 1951).

**California Sherry.** This wine, which is characterized by a n flavor, is the most important of the California wines, according to Cr The process, in brief, is as follows: White varieties of grapes (Palon Mission, Pedro Ximenes, etc.) are crushed and stemmed. The mus treated with sulfites in a concrete or wood vat. It is seeded with 2 per cent of yeast and fermented dry within a period of 5 to 8 days. pumped to a special room, where the alcohol content is ascertai Sufficient alcohol is added to bring the total amount to 20 per cent volume. Settling is permitted over a period of 1 day to 4 weeks. The w is pasteurized at 180 to 185°F., clarified with bentonite (8 to 10 lb. 1,000 gal.), and filtered. Baking, or cooking, is accomplished in barr tanks, or other containers, which are maintained at a temperature of ab 120°F. (occasionally higher) for approximately 3 months. Filter aid added to the wine, and it is filtered. Next it is cooled to 16 to 18°F. about 3 weeks, in order to hasten the precipitation of cream of tartar. is filtered again and aged in wood. Last, it is blended and bottl Sherries are pale to amber in color. Dry sherries usually contain 0 2.5 per cent sugar; medium sherries, 2.5 to 4 per cent sugar; and sw sherries, 4 to 7 per cent sugar.

**Correction of Deficiencies.** A number of substances may be added musts or wines to correct for deficiencies. Tartaric, citric, or malic a may be added to bring the acid to the desired level. Potassium tartr or calcium carbonate may be used to neutralize excess acidity. Tan at the rate of 0.125 to 1 lb. per 1,000 gal. may be incorporated in the m used to make white wine in order to facilitate clearing of the wine at early period. Pectolytic enzymes are used to aid in the clearing of jui or wines. Invert or cane sugar is added to wines to produce effervesce as a result of secondary fermentation in the container, bottle, or ta Grape must may be used before, during, or after the fermentation. Bran is used to arrest the fermentation in the making of appetizer and dess wines. Its use increases the alcohol content and volume of the wine which it is added. Substandard wines produced with too much water residue materials are used for distilling material in the making of bran

**Aging.** Reference has already been made to certain aspects of t important subject in connection with the making of red wine. Ag starts in wood and is completed in glass. During the process the w

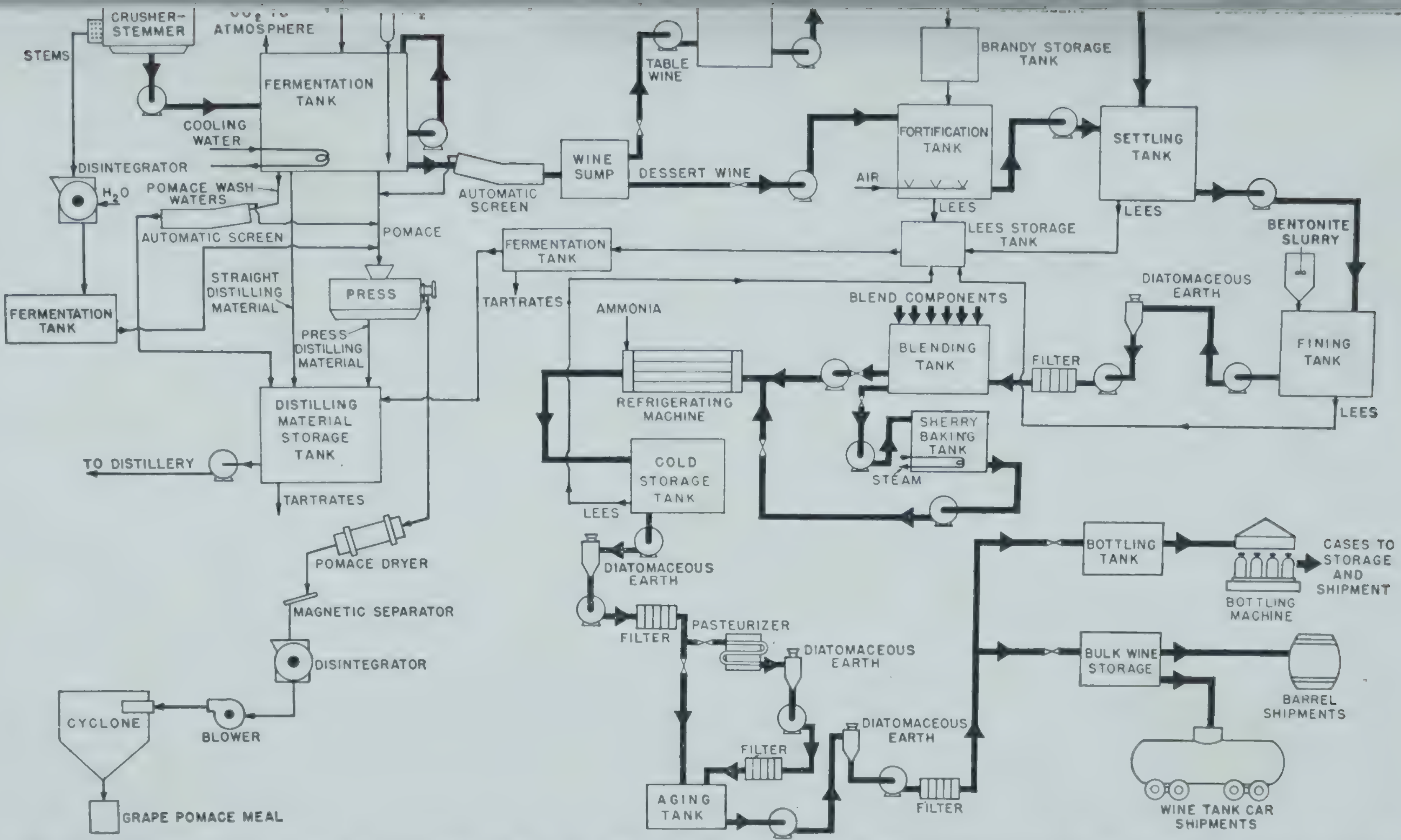

CRUSHER-STEMMER
STEMS
ATMOSPHERE
FERMENTATION TANK
COOLING WATER
DISINTEGRATOR
$H_2O$
POMACE WASH WATERS
AUTOMATIC SCREEN
FERMENTATION TANK
STRAIGHT DISTILLING MATERIAL
POMACE
PRESS
PRESS DISTILLING MATERIAL
DISTILLING MATERIAL STORAGE TANK
TO DISTILLERY
TARTRATES
POMACE DRYER
MAGNETIC SEPARATOR
DISINTEGRATOR
CYCLONE
BLOWER
GRAPE POMACE MEAL
AUTOMATIC SCREEN
WINE SUMP
TABLE WINE
DESSERT WINE
FERMENTATION TANK
TARTRATES
AMMONIA
REFRIGERATING MACHINE
COLD STORAGE TANK
LEES
DIATOMACEOUS EARTH
FILTER
PASTEURIZER
DIATOMACEOUS EARTH
FILTER
AGING TANK
DIATOMACEOUS EARTH
FILTER
BRANDY STORAGE TANK
FORTIFICATION TANK
AIR
LEES
LEES STORAGE TANK
BLEND COMPONENTS
BLENDING TANK
SHERRY BAKING TANK
STEAM
FILTER
DIATOMACEOUS EARTH
SETTLING TANK
LEES
BENTONITE SLURRY
FINING TANK
LEES
BOTTLING TANK
BOTTLING MACHINE
CASES TO STORAGE AND SHIPMENT
BULK WINE STORAGE
BARREL SHIPMENTS
WINE TANK CAR SHIPMENTS

FIG. 34. Modern wine making. [*Courtesy of W. Q. Hull, W. E. Kite, and R. C. Auerbach, Ind. Eng. Chem.*, 43(10): 2180 (1951). *Copyright 1951 by the American Chemical Society and reprinted by permission of the copyright owner.*]

mellows and forms bouquet, and changes take place affecting the fla odor, color, smoothness, clarity, and chemical composition. Specific there is an increase in the volatile acid content, esters, aldehydes, acetals and a decrease in the amount of tannins, coloring matter, fi acidity (owing to the deposition of potassium acid tartrate and the oc sional partial conversion of malic acid to lactic acid and carbon diox by fermentation), sugar, and certain other materials. Yeasts and bac rial cells, gums, unstable proteins, and other substances are deposited a removed by racking, filtration, and other procedures.

Aging is affected, according to Amerine (1950) by physical, biologi and chemical factors. The size and type of cooperage, temperatu amount of oxygen, humidity, and bottle size are physical factors of imp tance. Small containers favor greater exposure to oxygen, losses cau by evaporation and extraction of tannin, and woody flavors, but th effect easier clarification of wines and are desirable for small quantit of quality wines; however, they are undesirable for most red wines a all white wines. The importance of temperature has already been stress However, it should be pointed out that the use of higher temperatures f aging accelerates oxidative changes and extraction of tannin. Also el vated temperatures favor the growth of spoilage bacteria and quicken t darkening of wines—undesirable changes. Low temperatures inhib the development of spoilage bacteria and help to prevent oxidati changes from occurring in white wines.

As has been pointed out, oxidation of wines must be carefully co trolled, for excessive aeration results in the loss of bouquet and oth undesirable changes. But oxidation is desired in the case of sherries, f it favors the formation of aldehydes and other products which contribu to the flavor.

Ordinarily the growth of yeasts and bacteria in wine is highly und sirable. However, special yeasts are sometimes used in the making sherries; also, the partial conversion of malic acid to lactic acid an carbon dioxide through controlled fermentation may be beneficial.

Aging in glass softens the flavor, owing partly to a reduction in th tannin; causes a reduction in the amount of free sulfur dioxide (or i disappearance); removes the yeasty odor; and results in a gradual loss i bouquet. Temperature (optimum around 50 to 55°F. for table wines), th size of the bottle, the oxygen content of the wine when bottled, the siz and quality of the cork, the method of corking, the shape of the neck the bottle, the freedom of the cellar from vibrations, and lighting cond tions all affect the aging of wine in glass, according to Amerine (1950).

**Chemical Composition.** The characteristics of the finished win depend on the composition of the grapes used, the changes brought abou during fermentation and aging, and the contributions of special method of processing, such as in the making of sherry wine. Grapes supply th aroma; fermentation and aging supply the bouquet of wine.

Grapes contain 12 to 27 per cent of sugar (dextrose and levulose), 0.1 t 1.0 per cent of pectins, 0.1 to 0.5 per cent of pentosans, small amounts o arabinose, traces of xylose and inosite, and other related substances. Dr

|  | by volume |  |  | Balling at 68 F. |  |  | Total solids, g./100 ml. |  |  | g./100 ml. as dextrose |  |  | g./100 ml. as tartaric acid |  |  | g./100 ml. as acetic acid |  |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | Min. | Max. | Av. | Min. | Max. | Av. | Min. | Max. | Av. | Min. | Max. | Av. | Min. | Max. | Av. | Min. | Max. | Av. |
| White table wines: |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Sauterne (dry) | 11.0 | 13.8 | 12.0 | −2.2 | −1.4 | −1.8 | 1.7 | 2.8 | 2.1 | 0.05 | 0.30 | 0.17 | 0.45 | 0.78 | 0.62 | 0.040 | 0.100 | 0.060 |
| Sauterne (med. sweet) | 11.0 | 13.9 | 12.5 | 0.0 | 2.0 | 1.0 | 3.8 | 5.7 | 4.8 | 1.0 | 3.0 | 2.60 | 0.45 | 0.75 | 0.60 | 0.040 | 0.100 | 0.066 |
| Sauterne (sweet) | 11.2 | 13.9 | 12.8 | 1.5 | 3.0 | 2.5 | 5.4 | 7.5 | 6.6 | 3.0 | 5.8 | 4.6 | 0.45 | 0.75 | 0.60 | 0.040 | 0.100 | 0.060 |
| Rhine | 10.8 | 13.5 | 12.0 | −2.2 | −1.6 | −2.0 | 1.7 | 2.6 | 2.1 | 0.05 | 0.25 | 0.17 | 0.45 | 0.80 | 0.65 | 0.040 | 0.100 | 0.060 |
| Red table wines: |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Claret | 10.8 | 13.5 | 12.5 | −1.6 | −0.8 | −1.2 | 2.2 | 3.4 | 2.6 | 0.10 | 0.40 | 0.28 | 0.50 | 0.85 | 0.60 | 0.050 | 0.110 | 0.065 |
| Burgundy | 11.0 | 13.6 | 12.6 | −1.6 | −0.6 | −1.2 | 2.3 | 3.5 | 2.8 | 0.10 | 0.45 | 0.30 | 0.50 | 0.85 | 0.62 | 0.050 | 0.110 | 0.066 |
| Zinfandel | 10.9 | 13.5 | 12.4 | −1.6 | −0.6 | −1.2 | 2.2 | 3.5 | 2.7 | 0.10 | 0.40 | 0.28 | 0.50 | 0.80 | 0.59 | 0.050 | 0.110 | 0.069 |
| Dessert wines (white or amber): |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Muscatel | 19.5 | 20.5 | 20.0 | 5.5 | 10.0 | 6.5 | 11.7 | 16.0 | 12.6 | ..... | ..... | ..... | 0.30 | 0.50 | 0.42 | 0.025 | 0.070 | 0.040 |
| Angelica | 19.5 | 20.5 | 20.0 | 5.5 | 12.0 | 7.0 | 11.6 | 17.8 | 13.0 | ..... | ..... | ..... | 0.30 | 0.46 | 0.40 | 0.020 | 0.070 | 0.038 |
| White port | 19.5 | 20.5 | 20.0 | 5.5 | 8.0 | 7.0 | 11.5 | 14.0 | 13.0 | ..... | ..... | ..... | 0.30 | 0.44 | 0.38 | 0.020 | 0.060 | 0.035 |
| Sweet sherry | 19.5 | 20.5 | 20.0 | 3.0 | 7.0 | 6.0 | 9.1 | 13.4 | 12.0 | 6.6 | 11.4 | 9.8 | 0.35 | 0.55 | 0.46 | 0.035 | 0.065 | 0.048 |
| Dessert wines (red): |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Port | 19.5 | 20.5 | 20.0 | 5.5 | 9.0 | 6.5 | 11.6 | 15.0 | 12.6 | ..... | ..... | ..... | 0.38 | 0.65 | 0.50 | 0.035 | 0.070 | 0.050 |
| Tokay | 19.5 | 20.5 | 20.0 | 3.5 | 6.0 | 4.5 | 9.5 | 12.2 | 10.6 | ..... | ..... | ..... | 0.38 | 0.52 | 0.44 | 0.035 | 0.065 | 0.050 |
| Appetizer wines: |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Dry sherry | 19.5 | 20.5 | 20.0 | −3.2 | −2.2 | −2.5 | 3.0 | 4.3 | 3.9 | 0.5 | 2.4 | 2.0 | 0.32 | 0.58 | 0.48 | 0.030 | 0.070 | 0.050 |
| Sherry | 19.5 | 20.5 | 20.0 | −1.4 | −0.4 | −1.0 | 4.8 | 6.0 | 5.4 | 2.6 | 3.9 | 3.3 | 0.32 | 0.50 | 0.45 | 0.030 | 0.065 | 0.048 |
| Dry vermouth | 18.0 | 20.0 | 19.0 | −2.0 | 0.6 | −1.0 | 3.8 | 6.8 | 5.1 | ..... | ..... | ..... | 0.48 | 0.75 | 0.62 | 0.028 | 0.060 | 0.045 |
| Sweet vermouth | 16.0 | 19.0 | 18.0 | 9.0 | 13.0 | 11.0 | 14.5 | 18.5 | 16.8 | ..... | ..... | ..... | 0.45 | 0.70 | 0.60 | 0.030 | 0.060 | 0.045 |
| Sparkling wines: |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Champagne | 11.5 | 13.5 | 12.4 | −1.0 | 3.0 | 0.4 | 3.0 | 7.0 | 4.5 | 0.5 | 4.8 | 2.2 | 0.55 | 0.80 | 0.70 | 0.040 | 0.095 | 0.060 |
| Pink champagne | 11.5 | 13.2 | 12.4 | −0.4 | 1.5 | 0.0 | 3.6 | 5.6 | 4.1 | 1.2 | 3.3 | 2.0 | 0.55 | 0.76 | 0.68 | 0.040 | 0.095 | 0.063 |
| Sparkling Burgundy | 11.0 | 13.0 | 12.0 | 0.0 | 2.0 | 0.5 | 3.8 | 6.2 | 4.7 | 1.4 | 3.6 | 2.2 | 0.55 | 0.80 | 0.72 | 0.045 | 0.100 | 0.068 |

---

* W. Q. Hull, W. E. Kite, and R. C. Auerbach, *Ind. Eng. Chem.*, **43**(10): 2180 (1951). Copyright 1951 by the American Chemical Society and reprinted by permission of the copyright owner.

table wines contain very little sugar (refer to Table 55), whereas de
wines may contain up to 11 per cent or more of sugar. Wines also con
dextrins and other materials.

A number of alcohols are found in wines. Ethyl alcohol must be p
ent in table and sparkling wines in amounts below 14 per cent by volu
in dessert wines, in amounts over 18.5 per cent by volume (Win
1949). Higher alcohols, including amyl, isoamyl, *n*-butyl, *sec*-butyl,
butyl, and propyl, are found in concentrations of 10 to 175 mg. per 100
(Amerine, 1953). Glycerol may be present in amounts of 0.5 to 1.5
cent; 2,3-butanediol, in amounts of 0.01 to 0.15 per cent; and acetoin
amounts of 1 to 30 p.p.m. Diacetyl may be found in quantities of
4 p.p.m. in some red wines.

The acids in wines come from the grapes; arise during fermentat
aging, or special processing; or are added. Malic and tartaric acids
found in quantities of 0.1 to 0.5 per cent and 0.2 to 0.8 per cent, resp
tively, in musts, but the amounts of both are reduced during fermentat
and aging. Traces of citric acid and small amounts of tannic acid
originate in the grapes. Sulfurous acid is added to must or wine to ret
and prevent spoilage of a microbial nature. Formic, acetic, lactic,
propionic acid are produced during fermentation. The amounts of
first three of these acids especially may be increased as a result of
spoilage of the wine. Capric, caprylic, lauric, and *n*-butyric acids
reported to occur in wines (Winkler, 1949).

According to Amerine (1953), table wines ordinarily contain 0.5
1.0 per cent titrable acidity (calculated as tartaric). A total acidity
0.65 per cent, or higher, is desirable. The total acidity of dessert wine
normally lower and may desirably be in the range of 0.4 to 0.5 per ce
The maximum volatile acid content of wine (calculated as acetic)
0.120 g. per 100 ml. for white table and dessert wines and 0.140 g.
100 ml. for red table wines. An excessively high volatile acid content
regarded as an indication of spoilage.

Although wines often contain traces of a number of aldehydes, acetald
hyde is the most important one. In most wines the amount of acetald
hyde is low, but in some sherry wines there may be as much as 300
per liter.

Acetals are formed from aldehydes and alcohols. The acetal content
table wines may vary from 0 to 7 p.p.m.; that of dessert wines may
as high as 30 to 40 p.p.m. (Amerine, 1953).

Ethyl acetate is the most important ester found in wines. In a conce
tration of over 150 p.p.m. it may be objectionable. Other esters
formed in wines but appear not to be too important.

Tannins, which are astringent, may be found in white table a
sparkling wines in amounts of about 0.01 per cent, and in red table wi
in amounts ranging from 0.14 to 0.3 per cent.

Wines contain a number of other substances. Vitamins, especial
those of the B complex, are found in wines. Enzymes are present
unpasteurized wines.

**efects of Wine.** Defects of wine may be caused by microorganisms,
vhich case they are known as "diseases," or by other agencies. The
ases of wine are of two general types: those caused by aerobic micro-
anisms, and those caused by facultative anaerobes or anaerobes.

*iseases Caused by Aerobic Microorganisms.* The aerobic diseases of
es are caused principally by mycodermas and acetic acid bacteria.
se microorganisms grow well in wine in the presence of oxygen. They
se no trouble if the wine is carefully supervised during its manufacture
storage. They are most likely to become active during the fermenta-
of must, if the cap is not punched down frequently, and during the
age of wine, if the containers are not kept properly filled and sealed.
*andida mycoderma* (wine flowers) forms a film over the surface of
e and attacks the extract, the alcohol, and occasionally the organic
ls.

cetic acid bacteria will produce vinegar from wine in the presence of
gen, unless they are destroyed or prevented from growing. Their
vities are discussed in the chapter on Vinegar.

*iseases Caused by Facultative Anaerobes or Anaerobes.* TOURNE
ASE. The term "tourne" is considered to signify either the organism
ing the disease or the condition produced in wine by large numbers of
e bacteria. Tourne is considered to be the most serious disease of
es and one of the most common.

he organism is an anaerobic bacterium, which occurs as long, slender
. It may be found in red or white wines, or in fortified wines with an
hol content of 20 per cent or greater. It grows best, however, if the
hol concentration is not too great. Sugar and other nutrient sub-
ces favor its growth. It is likely to develop in wines "stuck" owing to
temperatures. It is inhibited slightly by tannin but very strongly
ulfur dioxide and metabisulfites.

urne is indicated by increasing volatile acidity, by decreasing fixed
ty, by a "silky" type of cloudiness, and, when the condition has
ressed well, by an odor and taste that is termed "mousey."

urne may be detected by a microscopic examination of the sediment,
ned by centrifuging a sample of the wine, or by analyses of the wine
olatile acids. If the maximum amount of volatile acid permitted in
by law—0.14 for red wine or 0.12 for white wine—has been produced
ceeded, then there is good evidence that the wine may be infected
tourne. Taste may also be of some assistance in its detection.

e judicious application of sulfur dioxide, 75 p.p.m., to wines, or
eurization; the use of a high degree of cleanliness about the plant;
lization of equipment with steam when necessary; and rigid labora-
control should lower the incidence, or prevent, tourne disease of wine.
pasteurization of bottled wine at a temperature of 145°F. for 30 min.
y effective in preventing tourne.

ce wine has been infected by tourne, it should be made brilliantly
by filtration with selected infusorial earths, or by clarification with
nite followed by passage through germproof filters. Sulfur dioxide,

or its equivalent of metabisulfite, should then be added to the wine in s quantity that its concentration will be maintained at 75 p.p.m., or grea All equipment that has been infected should be treated with live steam a suitable disinfectant to destroy the source of infection.

LACTOBACILLUS HILGARDII. Spoilage of some dry wines in Califor has been caused by *Lactobacillus Hilgardii,* a nonmotile, nonsporulat rod, which produces lactic and acetic acids. A silky cloudiness is p duced, while the flavor is affected and becomes somewhat "mousey."

MANNITOL- (MANNITE-) FORMING BACTERIA. Bacteria that prod volatile acid, lactic acid, and mannitol ($C_6H_{14}O_6$) from glucose grow w at temperatures above 100°F. Their growth is inhibited by keeping t temperature of fermentation well below this point, by the use of sulf dioxide, and by an increase in the acid content of the wine caused the addition of citric or tartaric acid.

SLIME-FORMING BACTERIA. Infected wine may become slimy and cloud Slime formation occurs chiefly in white wines, usually young wines closed containers. The occurrence of slime-forming bacteria in wine not common and may be prevented by adding tannin to wines low in th substance and by use of sulfur dioxide or metabisulfite.

OTHER BACTERIAL DISEASES. Cocci, which may be inhibited by $SO_2$ destroyed by pasteurization, may cause cloudiness in white wines.

Bitter wines may be caused by the growth of butyric acid bacteri while a sour wine may result from the growth of lactic acid bacteria.

*Defects Not Caused by Microorganisms.* Defects in wines may caused by metals, enzymes, and the improper use of certain finin agents.

Iron is a cause of clouding in wines. Two different types of defects a produced by iron: one is known as black, blue, or ferric casse; the oth as white casse.

*Ferric casse* is indicated by the appearance of a gray to gray-blue sedi ment in the wine and by clouding. The defect is found in bottled whit wines, especially. Iron forms a precipitate with the tannin and colorin matter of red wine. Only a few parts of iron in a million parts of win will produce ferric casse. Oxygen facilitates the formation of ferric ion from ferrous ions.

Ferric casse may be prevented by using equipment constructed of th proper types of metal and inhibited by 0.1 per cent citric acid.

The defect may be treated in one of several manners. In one method the iron is oxidized to the ferric stage by aeration. Tannin to the amoun of 0.05 per cent is added. Clarification with casein and bentonite follow In a second method, the iron is oxidized by aeration, tannin is added and then gelatin. Settling is permitted, which is followed by racking filtration, and acidification with citric acid. In a third method, tartari acid is added to the wine, and the wine is then refrigerated. Cream o tartar and iron salts are precipitated.

*White casse* is also caused by an excess of iron. The precipitate is du in part to iron phosphate. It occurs in white wine. Treatment is as outlined above for ferric casse.

*xidase casse* is uncommon. It is caused by an enzyme, oxidase, pro-
d by certain molds, which causes white wines to become brown
the color to be precipitated in red wines. Sulfur dioxide inhibits this
ase, while pasteurization at 180°F. destroys it.

*ther Defects.* Tin, tin salts, copper, aldehydes, or excessive quanti-
of gelatin may cause clouding of the wine. For a further discussion
his subject the reader is referred to the publications of Cruess and
r workers.

**oatings of Wine Tanks.** Concrete tanks are used for the fermenta-
of must and the storage of wine, but they must be lined to prevent
ndue amount of calcium from being dissolved by the wine. Steel
ks should be lined to prevent the solution of iron.

ccording to Cruess and his associates, one effective method for coat-
concrete is to treat the concrete first with a 0.5 per cent, then a
er cent. solution of tartaric acid. An insoluble calcium tartrate forms
the surface of the tank.

mixture of 25 per cent Gilsonite and 75 per cent paraffin produces a
ective coating for concrete and steel. Such linings are easily applied
not expensive.

ass-Hueter black enamel gives good results with both steel and con-
. A coating of paraffin over the enamel gives even better protection.

## Some References concerning Wine

, H. W.: "Natural Red Wines," Constable & Co., Ltd., London, 1951.
–: "Sherry and Port," Constable & Co., Ltd., 1952.
ine, M. A.: The Composition of California Wines at Exhibitions, *Wines &
ines*, **28**(1): 21–23, 42–43, 45; **28**(2): 24–26; **28**(3): 23–25, 42–46 (1947).
-: The Response of Wine to Aging, *Wines & Vines*, **31**(3): 19–22; **31**(4): 71–74;
1(5): 28–31 (1950).
-: The Composition of Wines, *Sci. Monthly*, **77**(5): 250–254 (1953).
- and M. A. Joslyn: Commercial Production of Dessert Wines, *Univ. Calif. Agr.
xpt. Sta., Bull.* 651, 1941.
and ———: "Table Wines, the Technology of Their Production in Cali-
rnia," University of California Press, Berkeley, Calif., 1951.
and L. B. Wheeler: A Check List of Books and Pamphlets on Grapes and
ine and Related Subjects, 1938–1948, University of California Press, Berkeley,
alif., 1951.
H. W.: Fermentation, Present Practices in the California Wine Industry, *Jour.
gr. Food Chem.*, **1**(2): 152–157 (1953).
, W. V.: "The Principles and Practice of Wine Making," 2d ed., Avi Publish-
g Company, New York, 1947.
- and C. R. Havighorst: How California Wines Are Made, *Food Inds.*, 1948,
p. 523–530.
man, H. J.: "Grossman's Guide to Wines, Spirits and Beers," Charles Scribner's
ons, New York, 1955.
k, U. P.: "Manual of American Grape Growing," The Macmillan Company,
ew York, 1919.
in, K. M., and T. C. Gregory: "Chemistry and Technology of Wines and
quors," D. Van Nostrand Company, Inc., Princeton, N.J., 1948.
V. Q., W. E. Kite, and R. C. Auerbach: Modern Winemaking, *Ind. Eng. Chem.*,
(10): 2180–2192 (1951).
H. E.: Grape Growing in California, *Univ. Calif. Coll. Agr. Ext. Ser., Circ.* 116,
rkeley, Calif., 1950. (Revised by A. J. Winkler.)

Lucia, S. P.: "Wine as Food and Medicine," McGraw-Hill Book Compan Blakiston Division, New York, 1954.

Melville, J.: "Guide to California Wines," Doubleday & Company, Inc., New 1955.

Schoonmaker, F., and T. Marvel: "American Wines," Duell, Sloan & Pearc New York, 1941.

Valaer, P.: "Wines of the World," Abelard Press, Inc., New York, 1950.

Wagner, P. M.: "American Wines and Winemaking," Alfred A. Knopf, Inc York, 1956.

Willig, M.: Wine Now Made Continuously, *Food Inds.*, 1950, pp. 1184–1185.

Wine Institute: A Selected List of Books on Wine, San Francisco, Jan. 30, 19

Winkler, A. J.: Grapes and Wine, *Econ. Botany,* 3(1): 46–70 (1949).

Ziemba, J. V.: Superior Quality Wines, *Food Eng.,* 1952, pp. 86–91, 188, 189.

## CHAPTER 8

# THE DISTILLING INDUSTRIES

he distilling industries are those concerned with the production of illed spirits: rum, whiskey, brandy, gin, vodka, cordials, and liqueurs.

**roduction Statistics.** Table 56 shows the production of distilled ts for the fiscal years ending June 30, 1936, 1946, and 1956. The key produced during the fiscal year 1956 represented 113,875,439 tax of bourbon whiskey, 12,655,734 tax gal. of corn whiskey, 2,959,392 gal. of rye whiskey, and 1,171,044 tax gal. of other whiskey.

TABLE 56. PRODUCTION OF DISTILLED SPIRITS [a]

| Distilled product | Tax gal. at registered and fruit distilleries | | |
|---|---|---|---|
| | 1936 | 1946 | 1956 |
| skey | 223,659,539 | 147,464,516 | 130,661,609 |
| dy | 20,094,419 | 34,416,385 | 8,545,215 |
| | 2,106,648 [b] | 2,657,580 [c] | 1,823,623 |
| | 6,229,241 | 7,497,402 [d] | 18,985,698 |
| ka | .......... | .......... | 6,542,062 |
| ts | 1,778,078 [e] | 113,030,754 [f] | 68,374,715 |
| tal | 253,867,925 | 305,066,637 | 234,932,922 |

ata obtained from U.S. Department of the Treasury, Internal Revenue Service, ol and Tobacco Tax Division, *Annual Reports of the Commissioner*, for the years ended June 30, 1936, 1946, and 1956, respectively.

ncludes 1,264,316 gal. produced at distilleries for denaturation.

ncludes 1,551,303 tax gal. removed for denaturation.

ncludes 7,005,916 tax gal. of spirits fruit.

ncludes 614,231 gal. produced at distilleries for denaturation.

roduction for beverage and industrial purposes.

he leading producing states during the fiscal year 1956 were as fol- : for whiskey, Kentucky (almost 70 per cent of the total), Illinois, ana, Maryland, and Pennsylvania; for brandy, California (about er cent of the total), Pennsylvania, New Jersey, Florida, and Oregon; um, Kentucky and Massachusetts (the only producing states); for New Jersey, Ohio, Illinois, Indiana, and New York; for vodka, Con- cut (over 50 per cent of the total), Ohio, Illinois, Kansas, and Massa- etts; and for other spirits, California (over 50 per cent of the total), ana, Iowa, Illinois, and Ohio.

The materials used in the production of distilled spirits during the f
year 1956 are indicated in Table 57. The grain and grain product
used for the production of whiskey, gin, vodka, and spirits; the mol
products are used for rum; and fruit and fruit products are used
brandy and spirits.

TABLE 57. MATERIALS USED IN THE PRODUCTION OF DISTILLED SPIRITS DURING THE FISCAL YEAR 1956 *

| Materials used at registered distilleries | Amount | Materials used at fruit distilleries | Amoun |
|---|---|---|---|
| Corn | 1,509,875,488 lb. | Grapes | 9,141,47 |
| Rye | 257,197,462 lb. | Raisins | 20,435,02 |
| Malt | 218,692,922 lb. | Other † | 2,254,94 |
| Wheat | 18,819,894 lb. | | |
| Sorghum grain | 110,516,248 lb. | Total | 31,831,44 |
| Barley | 7,740,218 lb. | Grapes | 365,414,807 |
| | | Raisins | 4,494,290 |
| Total | 2,122,842,232 lb. | Apples | 5,012,699 |
| Other materials ‡ | 959,153 lb. | Pears | 1,440,246 |
| Molasses | 2,805,726 gal. | Peaches | 293,658 |
| Other materials § | 5,147,844 gal. | Other ¶ | 44,933 |
| | | Total | 376,700,633 |

* Data obtained from U.S. Department of the Treasury, Internal Revenue Ser
Alcohol and Tobacco Tax Division, *Statistics Relating to the Alcohol and Tob
Industries for the Fiscal Year Ended June* 30, 1956, *Publication* 67, 1956.

† Represents 1,817,505 lb. pears, 230,190 lb. cherries, and 207,250 lb. apples.

‡ Represents 212,687 lb. diamalt, 39,810 lb. malt sirup, 3,252 lb. enzyme ext
670,664 lb. apples, and 32,740 lb. pears.

§ Represents 3,003,994 gal. enzyme extract, 2,031,437 gal. citrus waste concent
and 112,413 gal. pears.

¶ Represents 24,233 gal. cherries and 20,700 gal. blackberries.

## RUM

"Rum" is any alcoholic distillate from the fermented juice of sugarcane, su
cane sirup, sugarcane molasses, or other sugarcane by-products distilled at
than 190° proof (whether or not such proof is further reduced prior to bott
to not less than 80° proof) in such manner that the distillate possesses the ta
aroma, and characteristics generally attributed to rum; and includes mixtu
solely of such distillates.[1]

Rum is manufactured in general in those countries which grow su
cane or import molasses or other sugar-cane products. It is made pr
cipally in the United States, Puerto Rico, Cuba, Jamaica, St. Thomas a
St. Croix (Virgin Islands of the United States), British Virgin Islan
Demerara, Barbados, Uruguay, Martinique, Trinidad, Haiti, Sa

---

[1] U.S. Department of the Treasury, Federal Alcohol Administration, Regulati
5, Mar. 1, 1939.

mingo, the Leeward Islands, Guadeloupe, Mexico, Grenada, and
tigua.

**omposition.** Table 58 gives analyses of some rums from different
rces.

TABLE 58. ANALYSES OF SOME RUMS *

(Grams per 100 liters not calculated to proof)

| pH | Total acid (as acetic) | Volatile acid (as acetic) | Esters (as ethyl acetate) | Fusel oil (as amyl alcohol) | Solids | Aldehydes | Furfural | Color in 0.5-in. cell | Source, comments |
|---|---|---|---|---|---|---|---|---|---|
| 4.76 | 9.6 | 9.6 | 16.7 | 123.2 | 8 | 4.4 | 0.0 | 0.2 | Pennsylvania, new |
| 4.54 | 33.6 | 26.4 | 23.8 | 124.3 | 48 | 4.7 | Trace | 4.0 | Pennsylvania, 0.5 year old |
| 4.52 | 36.0 | 28.8 | 26.4 | 124.0 | 50 | 5.5 | 0.8 | 4.5 | Pennsylvania, 1 year old |
| 4.44 | 50.4 | 40.8 | 33.4 | 132.0 | 94 | 6.8 | 0.8 | 7.0 | Pennsylvania, 2 years old |
| 4.28 | 14.4 | 14.4 | 43.1 | 102.1 | 16 | 40 | 5.5 | 0.0 | Kentucky, new |
| 4.17 | 80.4 | 67.2 | 60.7 | 108.6 | 168 | 40 | 8.2 | 13.5 | Kentucky, 1 year old |
| 4.28 | 88.8 | 73.2 | 73.0 | 116.2 | 204 | 34.3 | 12.0 | 17.0 | Kentucky, 2 years old |
| 4.68 | 38.4 | 36.0 | 22.0 | 91.7 | 6 | 4.0 | 1.2 | 0.0 | Massachusetts, new |
| 4.42 | 84.0 | 74.0 | 29.0 | 91.5 | 86 | 6.4 | 1.2 | 7.5 | Massachusetts, 0.5 year old |
| 4.37 | 93.4 | 86.4 | 45.5 | 93.3 | 142 | 7.2 | 1.4 | 11.0 | Massachusetts, 1.5 years old |
| 4.30 | 98.4 | 86.4 | 51.0 | 96.8 | 162 | 7.2 | 1.6 | 13.0 | Massachusetts, 2 years old |
| 4.07 | 184.8 | 148.8 | 216.0 | 450.6 | 274 | 48 | 7.2 | 18.0 | Old New England, approx. 19 years old |
| 5.03 | 13.2 | 12.0 | 11.4 | 49.3 | 678 | 1.6 | 0.1 | 14.5, caramel | Cuba, Ron Bacardí Superior, gold |
| 8.05 | 4.8 | 2.4 | 44.0 | 59.8 | 205 | 7.0 | 0.8 | 15.5 | St. Croix cane juices, 2 years old |
| 4.17 | 57.6 | 48.0 | 59.8 | 91.5 | 450 | 20.0 | 4.0 | 23.0, caramel | Jamaica, 15 years old |
| 4.32 | 62.4 | 43.2 | 26.4 | 68.6 | 1,510 | 0.8 | 0.0 | 34.0, caramel | Demerara |

Valaer, Foreign and Domestic Rum, *Ind. Eng. Chem.*, **29**: 988 (1937). Copyright 1937 by the
can Chemical Society and reprinted by permission of the copyright owner.

um is a rather complex product, according to the researches of
yo (1945) and others. In addition to ethyl alcohol and water, it con-
varying amounts of glycerol, the higher monohydric alcohols, alde-
s (acetaldehyde, propaldehyde, furfural, etc.), esters (acetates,
ionates, formates, butyrates, isobutyrates, etc.), organic acids,
als, bases, and essential oils.

**ypes.** Rums made in the United States, with certain exceptions, may
assified as heavy-bodied rums (Valaer, 1937). They contain only the
enerics (acids, esters, etc.) that are accumulated during distillation
aging in charred white-oak barrels. Most of the rums are distilled at
oof less than 160 and aged for several years. They contain no color-
matter added by the distillers.

ums produced outside the United States, with exceptions, vary from
light to very heavy bodied. Cuba produces some of the lighest-
d rums, while Jamaica manufactures the heaviest-bodied rums out-
the United States. Substances are frequently added to such rums to
about the desired characteristics, one of these substances being
nel for artificial color (not all countries permit these additions,
ever).

Table 59 gives the analyses of some light-, medium-, and h bodied rums.

TABLE 59. ANALYSES OF SOME LIGHT-, MEDIUM-, AND HEAVY-BODIED RUMS

| | | G./100 liters of 100 proof | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Type of rum | Manufacture and location | Proof | Total acid (as acetic) | Esters (as ethyl acetate) | Aldehyde | Furfural | Fusel oil (as amyl alcohol) | Color | Wood extract | |
| Light bodied | Bacardí (Cuba), Carta Blanca [b] | 89.6 | 9.1 | 11.7 | 5.4 | 0.1 | 65.0 | Light | Slight | Bottled in the States |
| | Castillo (Cuba), Carta Oriente [c] | 87.8 | 9.7 | 7.0 | 7.3 | 0.1 | 79.0 | Dark | Slight | Bottled in the States |
| Medium bodied | Ron Rico (Puerto Rico), White Label [d] | 82.9 | 28. | 32. | 19. | 0.6 | 136. | Light | Present | Bottled in the States |
| | Bacardí (Cuba), "Extra Superior, 1873" [e] | 90.1 | 53. | 25. | 11. | 0.8 | 80. | Dark | Present | Bottled in the States |
| Heavy bodied | Wray's 3 Dagger (Jamaica) [f] | 99.8 | 34.0 | 56.0 | 18.0 | 4.0 | 238. | Dark | Present | For the States Bottle sample |
| | (Jamaica) for sale to Germany [g] | 149. | 141. | 565. | 19.0 | 5.4 | 114. | Dark | Present | High-pr From reled s |

[a] Courtesy of Walter C. Tobie.

[b] Typical of a very light flavored Cuban rum, such as is frequently used for highballs and co

[c] Although the color is dark, the flavor of this rum is light.

[d] An early sample (about 1934) for shipment to the United States.

[e] This is one of the heaviest Cuban rums. The flavor is relatively rich and fruity.

[f] A typical Jamaica rum for sale in the United States. The flavor is high.

[g] Bulk rum, said to have been prepared in Jamaica for the German trade. Note the excessivel ester value.

**Production of Rum.** *Fermentation.* The process for the manufa of rum is similar in many respects to that in which industrial alcoh produced. Methods for the propagation of pure cultures of yeast building up of a starter, the reaction of the main mash, the concentr of the fermentable sugar, the temperature of the fermentation, and the principal end products are similar. Some rums (Jamaica) are mac spontaneous fermentation, however.

In the manufacture of rum, the careful selection of the raw mate the control of the fermentation, proper distillation, and the aging o distilled product are essential for the production of a fine rum.

Strains of *Saccharomyces cerevisiae* or of other yeasts, such as sp of *Schizosaccharomyces*, may be used to "pitch" (inoculate) the n Great care must be exercised to maintain the selected yeast in culture, if the fermentation is to be successful.

As already indicated, rum is prepared from sugar-cane products the United States only blackstrap molasses is used in rum manufac This substance can be purchased at relatively low cost when proc arge quantities. It is a product of the sugar mill after the crys- zable sugar has been removed, and usually contains 40 to 55 per cent rmentable sugar.

concentration of molasses that contains 11 to 14 per cent of ferment- sugars may be used in preparing the main mashes. Ammonium te, and occasionally phosphates, may be added as a source of food he yeast. "Dunder," i.e., distillation slops which may be decomposed, be used to give the rum a heavier flavor and to supply nutrient erial.

lfuric acid is used to adjust the pH of the mash to 4.5 to 5.5, the r pH favoring a rapid fermentation and the production of a light- ored rum. The use of lower pH values favors slower fermentations the production of heavy-bodied rums, such as the Jamaica type. It sually necessary to add acid to the mash, unless it has been heat- ilized, to inhibit the development of undesirable bacteria.

he fermentation tanks must be cleaned carefully after being used if a n fermentation and light-bodied rum are desired. Steam, sulfuric acid, nonium bifluoride, or other agents may be used to prevent the devel- ent of undesirable flora in the tank.

fermentation temperature of about 70°F. (initial) to 96°F. (final) is sfactory. Low temperatures favor a slow fermentation, while at high peratures volatile constituents are lost. A range of 77 to 80°F. is quite rable.

lthough the main fermentation is nearly complete within 2 days, the hes are permitted to ferment for 3 to 7 days before being distilled. he fermented mash is sometimes designated as "beer."

*istillation.* Distillation is an important process in the production of Mashes that are distilled at high proof produce light-flavored rums, h are expected to be consumed shortly. Only small changes take in such products during storage, for the rums contain but small tities of acids, esters, fusel oil, and other congeneric substances. As d earlier, most rums in this country are distilled at a proof of less 160.

rious types of distillation equipment are used: pot stills, continuous- stills with combined rectifying columns, beer stills with doublers, bered stills, and other modifications.

the United States, rum distilled at a low proof is usually adjusted 0 proof before being aged.

*ging.* Aging is a process for improving the character of rum, for easing the undesirable flavors, and for greatly increasing the pleasant . Flavor, color, and aroma are developed, while the product is mel- d. During the first 6 months of the process, the main increases in s, acids, solids, and color take place. In a freshly distilled rum, the tity of esters usually exceeds that of the acids, but after storage ss than 6 months the ratio changes. Indeed, the acid content is usu- greater than the ester content after two years,[1] owing largely to the e products extracted from the wood of the barrel.

---

Valaer, Foreign and Domestic Rum, *Ind. Eng. Chem.*, **29**: 988 (1937).

The aging of rum is influenced by the following factors: the qualit the distillate (composition of nonalcoholic number), the proof, the na of the barrel (new oak ones are preferred), the temperature and rel humidity of the aging room, the action of oxygen, the time of aging, other factors.

Rum is usually aged in charred white-oak barrels, although som aged in plain cooperage. The aging is permitted to proceed for a months or for several years (in Government-bonded warehouses in United States).

Some rum is "quick-aged" by placing charred white-oak chips in rum before it is stored. Quick-aging may also be accomplished by ci lating the rum over oak chips. Heat and aeration are sometimes u Quick-aging processes are not particularly satisfactory, however.

**Government Supervision.** The manufacture of rum in the Un States and its possessions is carried out under Government supervis The quantity of raw material used and rum produced are carefully me ured. The Internal Revenue Service is interested in taxes, and the Fede Alcohol Administration in the promulgation of regulations concern standards for identification, such as labeling and advertising.

**Uses.** Rum may be used in the preparation of ice cream, candies, a mincemeat; in the curing of tobacco; as a beverage; and as a medicin

**Definitions and Regulations.** "New England rum" is rum that is produ in the United States, is distilled at less than 160° proof and is a straight rum a not a mixture of rums.

Puerto Rico, Cuba, Demerara, Barbados, St. Croix, St. Thomas, Vir Islands, Jamaica, Martinique, Trinidad, Haiti, and San Domingo rum are distinctive types of rum. Such names are not generic but retain their geograp significance. They may not be applied to rum produced in any other place th the particular region indicated in the name, and may not be used as a designat of a product as rum, unless such product is rum as defined.

Imitation rum—(1) neutral spirits or other distilled spirits which have be added thereto or which contain synthetic or imitation rum flavoring materi and (2) rum which has added thereto neutral spirits or other distilled spirits th rum are "imitation rum" and shall be so designated.[1]

## WHISKEY

According to Article II, Section 21, Class 2 of the Federal Alcoh Administration Act, Regulations 5,

> "Whisky" is an alcohol distillate from a fermented mash of grain distil at less than 190° proof in such manner that the distillate possesses the tas aroma, and characteristics generally attributed to whisky, and withdrawn fr the cistern room of the distillery at not more than 110° proof and not less th 80° proof, whether or not such proof is further reduced prior to bottling to n less than 80° proof; and also includes mixtures of the foregoing distillates f which no specific standards of identity are prescribed herein.

---

[1] U.S. Department of the Treasury, Internal Revenue Service, Federal Alcoh Administration, Regulations 5, as amended June 5, 1948.

**ypes.** There are several types of whiskey.

**omposition of Whiskeys.** Whiskeys contain approximately 50 per by volume ethyl alcohol. The flavor and bouquet are due mainly he impurities or congeneric substances that they contain, however. se accumulate during the production and storage of the whiskey. whiskey are found acids, esters, aldehydes, furfural, fusel oil, and ls, in addition to ethyl alcohol and water. The principal acids are ic and valeric acids,[1] but traces of propionic and other acids are d also. The combination of ethyl alcohol with these acids yields l acetate, ethyl valerate, and ethyl propionate, respectively, while amyl alcohols present in the fusel oil form amyl acetate, amyl rate, and other esters. Aldehydes include acetaldehyde and those lting from the oxidation of small amounts of some of the higher alco- during aging. Fusel oil is composed mainly of higher alcohols.

Table 60 are given the average analyses for 31 different samples rels) of whiskey, of which 14 were rye whiskey (13 being prepared by sweet-mash and 1 by the sour-mash method) and 17 were bourbon key (13 being prepared by the sour-mash and 4 by the sweet-mash od). The average data for the rye and bourbon whiskeys are also . The effect of aging on the quantities of the various components of key is illustrated. The results are expressed in grams per 100 liters, lated to the original volume of the whiskey. The acids are calcu- l as acetic acid, the esters and aldehydes as acetic, and the fusel oil nyl alcohol.

**oduction of Whiskey.** The flavor, aroma, and characteristics of the product depend on the nature of the raw materials and the fermenta- the method of distillation, and the aging process.

*Raw Materials.* Grains are the raw materials from which whiskeys roduced, the usual ones being rye, corn, wheat, and barley.

the United States, rye and bourbon are the principal types of whis- manufactured. Rye whiskey is generally prepared from rye and nalt or from rye and barley malt. A typical mash may contain r cent rye and 20 per cent barley malt. At least 51 per cent rye is red by law. Bourbon, on the other hand, is prepared from corn ze or Indian), barley malt or wheat malt, and usually another grain. ical mash may contain, for example, 70 per cent corn, 15 per cent nd 15 per cent malt; or 65 per cent corn, 23 per cent rye, and 12 per malt. The mash must always contain at least 51 per cent corn.

e preparation of a mash for whiskey is similar in fundamental ls to the preparation of a mash in the brewing industry, except that olids are not removed (Chap. 6). The enzymes of the malt convert tarches of the cooked grains to dextrins and sugars. At the same the protein molecules are degraded sufficiently to furnish the yeast a satisfactory source of nitrogen.

*Fermentation.* The resultant mash is fermented by a strain of ers' yeast, *Saccharomyces cerevisiae*, by either the sweet- or sour-

---

G. Woodman, "Food Analysis," 4th ed., McGraw-Hill Book Company, Inc., York, 1941.

TABLE 60. AVERAGE ANALYSES OF SOME WHISKEYS *

(Grams per 100 liters calculated to original volume)

| Age, years | Type | Color | Solids | Acids | Esters | Aldehydes | Furfural |
|---|---|---|---|---|---|---|---|
| New | Whole | .... | 20.0 | 6.4 | 15.0 | 4.03 | 0.71 |
| | Rye | .... | 13.6 | 4.7 | 13.7 | 4.91 | 0.97 |
| | Bourbon | .... | 26.0 | 7.7 | 17.2 | 3.26 | 0.44 |
| 1 | Whole | 7.3 | 101.5 | 37.8 | 29.9 | 7.08 | 1.5 |
| | Rye | 8.4 | 114.6 | 41.8 | 35.3 | 8.71 | 1.7 |
| | Bourbon | 6.4 | 90.1 | 34.4 | 24.9 | 5.55 | 1.3 |
| 2 | Whole | 8.6 | 124.2 | 46.1 | 42.9 | 8.34 | 1.7 |
| | Rye | 10.6 | 133.6 | 49.8 | 49.3 | 9.02 | 1.9 |
| | Bourbon | 6.7 | 114.8 | 42.7 | 37.3 | 7.78 | 1.4 |
| 3 | Whole | 10.2 | 140.2 | 51.1 | 48.4 | 9.47 | 1.8 |
| | Rye | 11.5 | 150.4 | 54.4 | 54.3 | 9.80 | 2.2 |
| | Bourbon | 8.3 | 130.7 | 47.8 | 42.5 | 9.15 | 1.5 |
| 4 | Whole | 10.2 | 140.4 | 51.6 | 50.9 | 10.2 | 1.9 |
| | Rye | 11.6 | 153.1 | 54.2 | 57.2 | 11.2 | 2.2 |
| | Bourbon | 8.9 | 127.7 | 48.9 | 45.0 | 9.3 | 1.5 |
| 5 | Whole | 11.1 | 149.2 | 52.2 | 51.1 | 10.2 | 1.9 |
| | Rye | 12.2 | 158.8 | 54.8 | 57.5 | 11.3 | 2.5 |
| | Bourbon | 10.0 | 140.2 | 49.8 | 45.0 | 9.2 | 1.5 |
| 6 | Whole | 11.1 | 151.4 | 53.2 | 50.7 | 10.2 | 1.9 |
| | Rye | 12.3 | 161.0 | 54.8 | 55.5 | 11.3 | 2.4 |
| | Bourbon | 10.1 | 142.5 | 51.8 | 45.2 | 9.1 | 1.4 |
| 7 | Whole | 11.1 | 154.0 | 52.2 | 51.1 | 9.8 | 1.8 |
| | Rye | 12.0 | 161.3 | 51.9 | 56.6 | 10.6 | 2.2 |
| | Bourbon | 10.2 | 147.1 | 52.4 | 46.4 | 9.0 | 1.4 |
| 8 | Whole | 10.5 | 155.2 | 53.1 | 50.9 | 9.6 | 1.8 |
| | Rye | 11.1 | 163.8 | 52.6 | 56.7 | 10.6 | 2.2 |
| | Bourbon | 10.0 | 147.7 | 53.6 | 45.9 | 8.8 | 1.5 |

* C. A. Crampton and L. M. Tolman, *Jour. Am. Chem. Soc.*, **30**: 98 (1908). C right 1908 by the American Chemical Society and reprinted by permission of copyright owner.

mash method. In the former method, which is commonly used, the m is inoculated directly with the yeast. Less time is required for this mentation, generally, and a higher yield of alcohol is obtained than f fermented sour mashes. In the latter method the spent slops and "ba (yeast) from tanks previously set and fermented are mixed with mash before fermentation.

Bacteria play a part in the production of esters in whiskey manu ture, the temperatures attained during fermentation favoring their grow Not infrequently the distiller may grow his selected culture of yeast i medium containing lactic acid bacteria, the lactic acid favoring

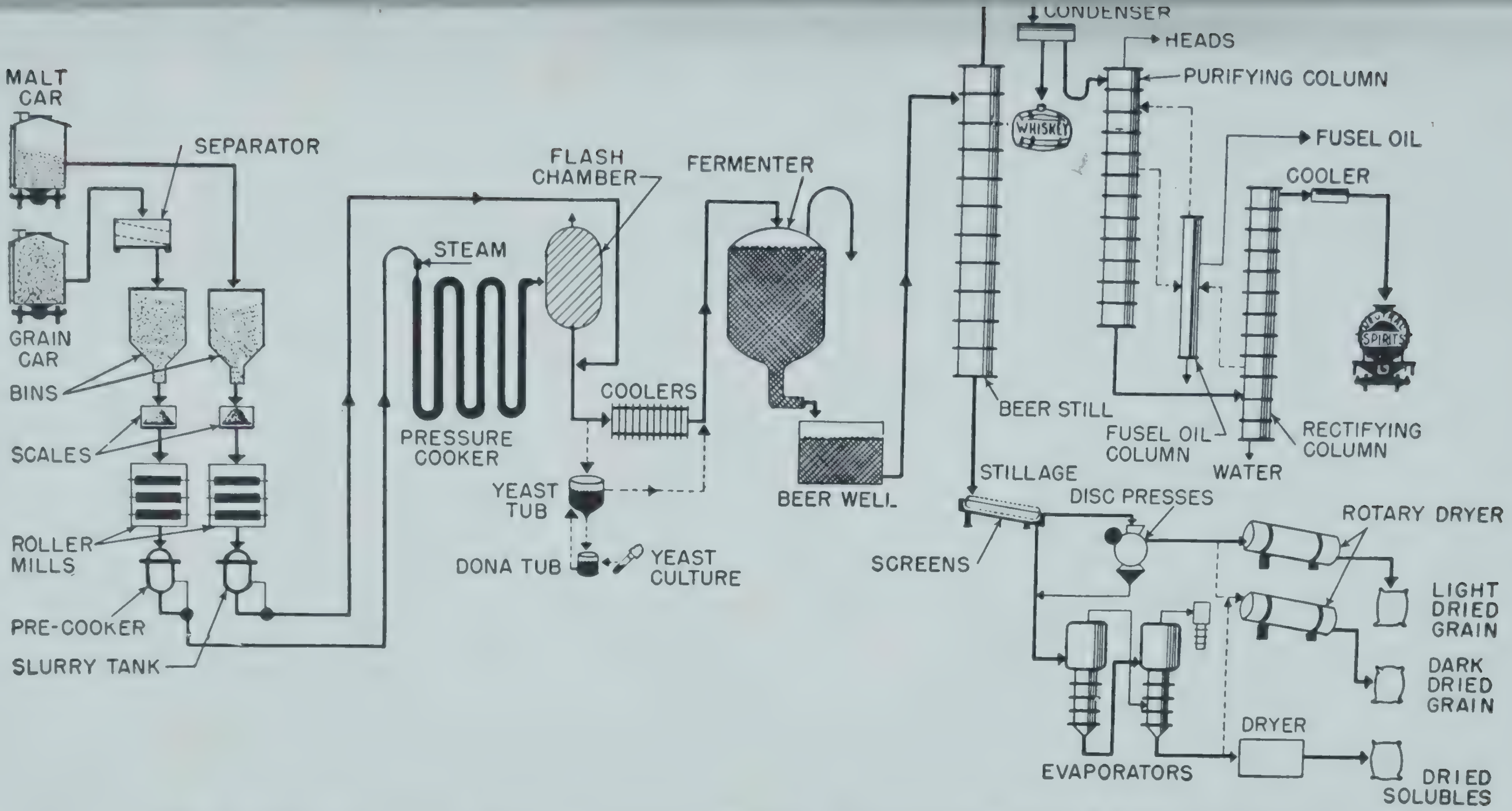

MALT CAR
GRAIN CAR
SEPARATOR
BINS
SCALES
ROLLER MILLS
PRE-COOKER
SLURRY TANK
STEAM
PRESSURE COOKER
FLASH CHAMBER
COOLERS
YEAST TUB
DONA TUB
YEAST CULTURE
FERMENTER
BEER WELL
CONDENSER
HEADS
PURIFYING COLUMN
WHISKEY
FUSEL OIL
COOLER
SPIRITS
BEER STILL
FUSEL OIL COLUMN
WATER
RECTIFYING COLUMN
STILLAGE
SCREENS
DISC PRESSES
ROTARY DRYER
LIGHT DRIED GRAIN
DARK DRIED GRAIN
EVAPORATORS
DRYER
DRIED SOLUBLES

FIG. 35. Flow diagram of whiskey production. (*Courtesy of Joseph E. Seagram & Sons, Louisville, Ky.*)

growth of yeast, inhibiting certain undesirable types of microor and contributing to the aroma, flavor, and characteristics of the w

3. *Distillation.* Both the type of still and the method of dis are important, for they contribute to the quality of the produ continuous still, with doubler, and the three-chambered still, wi bler, are two commonly used stills.

4. *Aging.* During the process of aging, definite changes tak in certain of the congeneric substances of whiskey as the result of it in oak containers. These changes have been investigated i detail by Crampton and Tolman (1908) and by Valaer and (1936) and discussed by Liebmann (1953).

"Age" for "American type whiskeys, other than corn whiskey, s corn whiskey, blended corn whiskey, and blends of straight corn w produced on or after July 1, 1936, means the period during wh whiskey has been kept in charred new oak containers."[1]

Whiskey is stored in heated or unheated warehouses for aging causing greater extraction of substances from the wood of the con

CHANGES DURING AGING. During aging in white-oak barrels, c take place in the solids, esters, acids, fusel oil, aldehydes, furfur color, the largest increase in solids, esters, acids, and color oc during the first half year of storage (Valaer and Frazier, 1936).

The solids of whiskey, known also as "extract," are derived fr wood of the container. During the first 6 months of storage, and Frazier found an average increase of about 70 g. of solids p liters. The quantity of extract was progressively smaller during eac ceeding 6-month period, being apparently 4 g. per 100 liters duri period between the forty-second and forty-eighth months of storage

Since there is very little change in the ester content of a whiskey quick-aging, the determination of esters may be used as a depe index of the age of the whiskey.

The acids of whiskey are both volatile and fixed acids. The part of the increase in total acids during storage is due to volatile Some fixed acids are extracted from the wood of the container.

During storage in charred oak barrels, there is an actual loss quantity of fusel oil, according to Valaer and Frazier. The fu determination gives information concerning the method of disti and is, therefore, considered to be an important determination.

There is an actual loss of aldehydes during storage.

Some furfural is extracted from charred oak barrels during th 6 months of storage, but very little appears later. Uncharred barr not contribute furfural.

QUICK-AGING. The process of quick-aging of whiskey is used sively, especially for whiskeys that are to be marketed shortly after ufacture. Quick-aging produces but little change in the ester conte no effect on the fusel oil. It increases the aldehydes only slightly. acids (mainly the nonvolatile ones), furfural, and color are inc

---

[1] U.S. Department of the Treasury, Internal Revenue Service, Federal Administration, Regulations 5, as amended to Mar. 1, 1939.

r depth is readily varied by means of this process. The following
illustrates the effect of quick-aging on a whiskey.

eat, charred or uncharred wood chips, and charred barrels are used
me of the quick-aging processes.

ANGES IN GLASS. Although extensive changes do not usually occur
hiskeys stored in glass containers, evidence indicates that there may
slight decrease in acids in the case of some whiskeys, especially those
h are not new. Valaer and Frazier (1936) suggest that this reduction
idity may be due to the alkali dissolved from the glass, to the estab-
nent of a change in equilibrium, or to an oxidation change. Esters tend
crease, as does the color, while furfural is reduced in quantity.

TABLE 61. DIFFERENCE IN SAME WHISKEY WITH AND WITHOUT QUICK-AGING *

| | G./100 liters calculated to proof | |
|---|---|---|
| | Quick-aged | Not quick-aged |
| Total acids | 11.9 | 5.9 |
| Esters | 14.3 | 15.6 |
| Fusel oil | 124.3 | 127.6 |
| Solids | 33.6 | 11.2 |
| Color | 3.5 | 0.0 |
| Furfural | 1.0 | 0.0 |

. Valaer and W. H. Frazier, *Ind. Eng. Chem.*, 28: 92 (1936). Copyright 1936 by
American Chemical Society and reprinted by permission of the copyright
.

**deral Control.** The manufacture of whiskey is carried out under
rnment supervision from the weighing of the grain to the sale of the
key. The Internal Revenue Service and the Federal Alcohol Admin-
ion Division are much interested in the control of the whiskey pro-
on. For details in connection with importing, bottling, labeling, and
tising, the reader is referred to the publications of the latter division,
of which are listed in the references given at the end of this section.

**ndards of Identity.** Standards of identity for distilled spirits are
nted in Regulations 5, Relating to Labeling and Advertising of Dis-
Spirits, as amended June 5, 1948, U.S. Department of the Treasury,
ol and Tobacco Tax Division, *Publication* 4, February, 1957.

## References on the Distilling Industries

### RUM

, R.: Studies on Rum, *Univ. Puerto Rico Agr. Expt. Sta., Research Bull.* 5,
io Piedras, P.R., December, 1945.
W. C.: Improved Procedures in Determination of Aldehydes in Distilled
coholic Liquors with Schiff's Reagent, *Food Research*, 3: 499 (1938).
: Personal communication, 1939.

U.S. Department of the Treasury, Federal Alcohol Administration, Regulati
Mar. 1, 1939.

———: Notice to Bottlers and Importers of Distilled Spirits, Feb. 28, 1936.

Valaer, P.: Foreign and Domestic Rum, *Ind. Eng. Chem.*, **29**: 988 (1937).

## Whiskey

Adams, A. B.: The Distillation of Whiskey, *Jour. Ind. Eng. Chem.*, **2**: 34 (1910)

Anonymous: A Modern Distillery, *Chem. Eng.*, 126-II–129-II, November, 1942.

Anonymous: "Plant Operations," The Calvert Distilling Co., Louisville, Ky., 194

Association of Official Agricultural Chemists: "Official and Tentative Metho
Analysis," 6th ed., Washington, 1945.

Boruff, C. S.: "Microbiology in a Modern Whiskey Distillery," Abstracts of C
munications, Third International Congress for Microbiology, September, 1
p. 333.

Brown, T. F.: Whiskey Distilling, *Am. Wine & Liquor Jour.*, September, 1937.

——— and H. H. Schopmeyer: The Production of Bourbon, *Am. Wine & Liq
Jour.*, December, 1935, p. 54.

Crampton, C. A., and L. M. Tolman, *Jour. Am. Chem. Soc.*, **30**: 98 (1908).

Foth, G.: "Handbuch der Spiritusfabrikation," Paul Parey, Berlin, 1929.

Gallagher, M., P. Kolachov, and H. F. Willkie: Whiskey Losses during Ag
*Ind. Eng. Chem.*, **34**: 992–995 (1942).

Hirsch, I.: Manufacture of Whiskey, Brandy and Cordials, 2d ed., Sherman E
neering Co., Newark, N.J.

Kolachov, P.: The Distilling Industry in War and Peace, *Trans. Am. Assoc. Cer
Chemists*, **2**(3): 14–23 (1944).

Owen, W. L.: The Amylo Process of Malting in Whiskey Distilleries, *Am. Wine
Liquor Jour.*, June, 1936.

Schicktanz, S. T., and A. D. Etienne: Acid Content of Whiskey. Variation with A
and Dilution, *Ind. Eng. Chem.*, **29**: 157–159 (1937).

Schmitt, R. O., P. J. Kolachov, and H. F. Willkie: Processing Equipment
Whiskey Filtration, *Ind. Eng. Chem.*, **34**: 1135–1138 (1942).

Unger, E. D.: Recent Advances in Alcohol Distillation Methods, *Chem. Inds. Wee*
May, 1946, pp. 778–780.

U.S. Department of the Treasury, Federal Alcohol Administration, Relating
Labeling and Advertising of Distilled Spirits, Regulations 5, as amended
Mar. 1, 1939, 1939.

———: Regulations 5, Amendment 5, Feb. 28, 1938; Amendment 6, June 8, 1938.

———: Notice to Bottlers and Importers of Distilled Spirits, Feb. 28, 1936.

———: A Digest of Interpretations of Regulations 5 Relating to Labeling and Adve
tising of Distilled Spirits, January, 1937.

Valaer, P., and W. H. Frazier: Changes in Whiskey Stored for Four Years, *Ind. E
Chem.*, **28**: 92 (1936).

Willkie, H. F.: Distillery Processes, *Chem. Eng.*, May, 1945.

Woodman, A. G.: "Food Analysis," 4th ed., McGraw-Hill Book Company, In
New York, 1941.

## General

Barron, H.: "Distillation of Alcohol," Joseph E. Seagram & Sons Co., Inc., Lou
ville, Ky., 1944.

Beavens, E. A., H. E. Goresline, and E. K. Nelson: Silver in the Artificial Aging
Brandies, *Ind. Eng. Chem.*, **29**: 623 (1937).

Goresline, H. E.: "List of Selected References on Distilled Liquors and Alcohol
U.S. Department of Agriculture, Bureau of Agricultural and Industrial Che
istry, Food Research Division, July, 1936.

Guymon, J. F., N. E. Tolbert, and M. A. Amerine: Studies with Brandy. I. P
*Food Research*, **8**(3): 224–230 (1943).

n. K. M., and T. C. Gregory: "Wines and Liquors," D. Van Nostrand Com- ny, Inc., Princeton, N.J., 1935.

n. E.: "Handbuch für die Getranke-industrie, Getranke-fabriken und den tranke-gross-betrieb," Paul Parey, Berlin, 1925.

M. A., and M. A. Amerine: Commercial Production of Brandies, *Univ. Calif. r. Expt. Sta., Bull.* 652, Berkeley, Calif., September, 1941.

nn, A. J.: Distilled Alcoholic Beverages, *Jour. Agr. Food Chem.*, 1(19): 1146– 2 (1953).

r, C.: "Distillation et rectification des liquides industriels," Dunod, Paris, 5.

W. C.: 1/10 of a degree for accurate proofing, *Am. Wine & Liquor Jour.*, ril, 1939, p. 17.

, N. E., M. A. Amerine, and J. F. Guymon: Studies with Brandy. II. Tan- , *Food Research,* 8(3): 231–236 (1943).

## Some Patents

, L. H., and R. H. Harlin: Apparatus for Purifying and Aging Liquor, U.S. ent 2,108,661, Feb. 15, 1938.

, G. A.: Process for Artificially Aging Alcoholic Liquids and Perfumes, U.S. tent 2,109,151, Feb. 22, 1938.

nn, A. J.: Method of Making Gin, U.S. Patent 2,152,318, Mar. 28, 1939.

C. D.: Aging Device for the Contents of Barrels, Casks, or the Like, U.S. ent 2,135,622, Nov. 8, 1938.

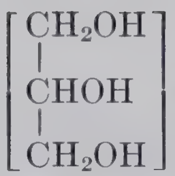
, C. K.: Aging Distilled Alcoholic Spirits, U.S. Patent 2,132,435, Oct. 11, 1938

, A.: Process of Making Liquors, U.S. Patent 2,160,036, May 30, 1939.

, C. J.: Method of Producing Yeast Mash, U.S. Patent 2,132,091, Oct. 4, 1938.

## CHAPTER 9

# THE GLYCEROL FERMENTATION

Glycerol  $\left[\begin{array}{c} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{array}\right]$ is the simplest alcohol that contains three hydro groups. It is a chemical substance that has many uses in industry and the arts.

In industry, glycerol is prepared by the saponification of fats an oils in the making of soaps, whereas synthetic glycerol may be mad from propylene or propane. A very small amount of free glycerol i found in palm and in some less common oils. During World War I approximately 1,000 tons of glycerol per month were manufactured by fermentation method, known as the "sulfite process" (Lawrie, 1928).

**Historical.** In his studies of wines and beers, Pasteur found that glycerol was formed regularly by yeasts to the extent of 2.5 to 3.6 per cen on the basis of the weight of the sugar fermented.

About three years before the beginning of World War I, Neuberg an his fellow workers commenced to publish some of the results of thei studies concerned with the mechanism of the ethyl alcohol fermentatio by yeasts. Neuberg's scheme for the course of events during the norma fermentation is shown in Chap. 5. While experimenting on aldehyd fixation with sodium sulfite in an attempt to elucidate further facts concerning the mechanism of the fermentation, he discovered that a larg increase occurred in the amount of glycerol formed when sulfite wa added to the fermentation medium. He suggested the following reaction

$$C_6H_{12}O_6 + Na_2SO_3 + H_2O \rightarrow NaHCO_3 + CH_3{\cdot}CHO{\cdot}NaHSO_3 + C_3H_8O_3$$

From the preceding equation, 100 g. of hexose theoretically yields 51 of glycerol when 70 g. of anhydrous sodium sulfite is used. At the sam time, slightly more than 24.4 g. of acetaldehyde is fixed. The ratio o acetaldehyde to glycerol is 24.4:51, or 1:2.09.

Table 62 shows the yields of glycerol and aldehyde obtained when th proportions of sodium sulfite were varied. An examination of this tabl indicates that the ratio of aldehyde to glycerol that was obtained wa fairly close to the theoretical and independent of the quantity of sulfit used.

Neuberg's second and third schemes indicate the courses of the fermentations when a sulfite and an alkaline medium are used.

62. Effect of Various Amounts of Sodium Sulfite on Yields of Glycerol and Aldehyde *

| $_2SO_3$, s used | Sugar, parts used | Aldehyde, parts produced | Glycerol, parts produced | Ratio of aldehyde to glycerol |
|---|---|---|---|---|
| 33 | 100 | 11.90 | 23.37 | 1:1.96 |
| 50 | 100 | 12.52 | 24.86 | 1:1.98 |
| 75 | 100 | 13.89 | 27.62 | 1:1.98 |
| 150 | 100 | 18.65 | 36.90 | 1:1.98 |

W. Lawrie, "Glycerol and Glycols," Reinhold Publishing Corporation New 1928.

**ethods.** Increased yields of glycerol may be secured by the use of , by acid salts, by neutral salts, by alkalies, or by alkaline salts.

ree well-known processes for the production of glycerol by fermenta- are the sulfite process, with which the names of Connstein and cke are associated and which was used on a large scale by Germany ig World War I; the Cocking and Lilly process, in which a mixture lfite and bisulfite is used; and the alkaline process developed by in the United States.

all these processes the basic medium contains a fermentable sugar if necessary, added nutrient salts. The medium is inoculated with ast culture and maintained at the optimum temperature for the fer- ation, usually 30 to 37°C. From time to time during the fermenta- small portions of the agents that cause increased yields of glycerol dded to the mash. The normal course of the fermentation is altered.

cerol may also be produced by certain osmophilic yeasts (refer to . 11) and by *Bacillus subtilis* (Ford's strain) (Chap. 22).

**e Sulfite Process.** The basis for this process is the fixation of ldehyde by sodium sulfite:

$$CH_3 \cdot CHO + Na_2SO_3 + H_2O \rightarrow CH_3 \cdot CHO \cdot HSO_3Na + NaOH$$

rmally acetaldehyde is reduced in large part to ethyl alcohol during rmentation of sugars by yeasts. But when this hydrogen acceptor nes fixed by sulfites, a second molecule in the mash, a triose pro- from hexose, acts as the main hydrogen acceptor and becomes ed to glycerol (see page 134 of Chap. 5 for the reactions).

e effect of various concentrations of sodium sulfite on the glycerol is shown in Table 63.

reasing the amounts of sodium sulfite caused a corresponding ise in the quantity of fixed aldehyde and decreases in the yields of ol and carbon dioxide.

s giving an alkaline reaction were found to increase the yield of ol, but alkaline media favored the growth of contaminants. By large amounts of sodium sulfite, the danger of infection was ated.

sulfite process that the Germans used during World War I was

Table 63. Effect of Various Concentrations of Sodium Sulfite on Y
of Glycerol *

| Sodium sulfite, parts by weight | Sugar, parts by weight | Glycerol yiel based on sug |
|---|---|---|
| 40 | 100 | 23.1 |
| 67 | 100 | 24.8 |
| 80 | 100 | 27.3 |
| 100 | 100 | 30.1 |
| 120 | 100 | 33.0 |
| 150 | 100 | 34.6 |
| 200 | 100 | 36.7 |

* J. W. Lawrie, "Glycerol and Glycols," Reinhold Publishing Corporati
York, 1928.

founded on the following example (May and Herrick, 1930).
(1 kg.), nutrient salts (50 g. of ammonium nitrate and 7.5 g. of
sium phosphate), and sodium sulfite (400 g.) were dissolved in w
liters) and inoculated with fresh yeast (100 g.), the mash being p
to incubate at 30°C. for 2 to 2.5 days.

Although beet sugar was used by the Germans in the sulfite
it has been stated that "neither the kind of sugar nor the va
yeast influence the fermentation" (Lawrie, 1928). Yeast may
repeatedly, if purified between fermentations.

It is important to control the temperature of the fermentatio
cially during the summer months.

Glycerol may be recovered by the following method of Connst
Lüdecke: Alcohol and acetaldehyde are separated by distillati
sulfite in the spent slop is precipitated as calcium sulfite by the
of calcium oxide, hydroxide, or chloride, and filtered out. Calci
that remain in the filtrate are treated with sodium carbonate to f
insoluble calcium carbonate, which is removed. Technically pure
is obtained by distilling the liquor, which contains glycerol and
chloride mainly, under reduced pressure.

In order to determine the amount of glycerol present in the m
filtrate from which the carbonates have been removed may be
to a sirup by evaporation and then extracted with ethyl alcohol.
and alcohol are separated by the process of evaporation. The
may then be determined by the Zeisel-Fant isopropyl iodine m
distilled and weighed as such.

For an extended discussion of the recovery and determin
glycerol, in which there are certain problems, the interested r
referred to "Glycerol and Glycols," by J. W. Lawrie.

Theoretically, a yield of 51 parts of glycerol should be obtain
100 parts of hexose. Yields of this magnitude are not obtained
sulfite process, but considerable of the glycerol is lost owing
efficiency in the recovery process. On the basis of the weight of

nted, approximately 20 to 25 per cent of glycerol, 30 per cent of ol. and 5 per cent of acetaldehyde were obtained by Connstein and cke. Actually it required usually 10 to 12 kg. of refined sugar to ice 1 kg. of dynamite glycerol by this process on an industrial basis.

**e Cocking-Lilly Process.** This process is a modification of the sul- rocess of Connstein and Lüdecke. Mixtures of normal sulfites and fites of the alkali metals are added to the fermenting mash. The ntation time is much shorter than in the normal sulfite process. s should be higher.

e addition of bisulfites to a fermenting mash causes the acetaldehyde fixed at an earlier stage in the fermentation than is usually possible ids in neutralizing the bicarbonate formed.

ulfites are antiseptic in nature. Owing to their antiseptic properties, cannot be used alone in the fermentation mash in large amounts. hey may be mixed with normal sulfites to produce a combination is neutral to litmus and which does not demonstrate too strongly itory action toward the yeast.

bisulfite is formed when sodium sulfite is added to a fermenting , but it reacts with acetaldehyde to form the fixation product:

$$Na_2SO_3 + H_2O + CO_2 \rightarrow NaHSO_3 + NaHCO_3$$

ien a mixture of sulfites (molecule for molecule) is added to fer- ing media, the reaction is as follows:

$$Na_2SO_3 + NaHSO_3 + CO_2 + H_2O \rightarrow 2NaHSO_3 + NaHCO_3$$

ring the fermentation, an aqueous solution of a bisulfite (preferably m) is added in successive small portions, or aqueous solutions of a re of sulfites, containing increasing proportions of bisulfite, are as the fermentation proceeds. The relative proportions of the sul- hould be such that the combination is neutral or approximately l to litmus.

total quantity of sulfites to be added to a mash may be calculated on the basis of the yield of glycerol desired or on the basis of the tical yield of glycerol.

**dies of Freeman and Donald.** In a study of the influence of vari- on the formation of glycerol from Cuban blackstrap molasses in the ce of sulfites, Freeman and Donald (1957*a*) found the following um conditions: an initial reducing sugar concentration of 20 to 22 g. 0 ml., a maximum free sulfite concentration of 3 to 3.5 g. per 100 ml. $O_3$ equivalent), the restriction of aeration with air to 30 min. during refermentation" period, a pH of 6.7 to 7, and a temperature of 35°C. Under these conditions and with a sulfite dosage of 40 per he fermentation was complete in 5 days and yields of 27.2 per cent ol, 10 per cent acetaldehyde, 21 per cent ethanol, and 1.1 per cent acid were obtained.

mixture of sulfite and bisulfite strongly inhibited the growth of *romyces cerevisiae* in unaerated media (Freeman and Donald, ). No growth of the yeast occurred at a concentration exceeding

3.1 g. per 100 ml. and practically none in the range of 2 to 3.1
100 ml. Maximum viability of the yeast was found in the pH ra
8 to 8.5 Acetaldehyde was more toxic to the yeast than the acetald
sodium sulfite compound. Acclimatization of the yeast strains le
apparent advantages in the fermentation.

**The Eoff Process.** This process, one for producing glycerol
alkaline medium, was developed by Eoff, Linder, and Beyer (19
the Division of Chemistry of the Bureau of Internal Revenue. Inv
tions were initiated in 1917 as a result of reports that glycerol was
made by fermentation methods in Germany.

*The Process in Brief.* A nutrient solution containing a sugar is
lated with a selected "trained" yeast and incubated at 30 to 32°
alkaline reacting compound is added to the fermenting medi
amounts up to approximately 5 per cent, in accordance with a d
schedule. The fermentation proceeds usually from 5 to 7 days
the glycerol is recovered.

*Details of the Process.* Blackstrap molasses, solutions containing
tures of corn sugar and malt sprouts, and solutions of sucrose cont
nutrient salts have been used successfully as media. The optimum co
tration of sugar is 17.5 to 20 g. per 100 ml. Ammonium chloride in
amounts aids in the production of glycerol.

Two yeasts were found to produce the highest yields of gly
*S. ellipsoideus* (var. Steinberg) and *S. ellipsoideus* (var. California
yeast) (varieties of *S. cerevisiae*), the former yeast giving the
results.

Yeast that has been "trained" or acclimated to growth in an alk
medium produces the highest yields of glycerol. The main mash is i
lated with a starter that represents approximately 10 per cent
volume. The first culture of yeast may be grown in a small flask. T
ing is given to the yeast by adding 0.5 to 1 per cent of sodium carb
(calculated on the basis of the weight of the solution) to the culture.
first effect is to stop fermentation. This is transitory. When the fer
tation becomes active again, a larger volume of mash is inoculated
5 to 10 per cent of trained yeast. Alkali is added to this mash, and
the culture becomes active a proportionally larger mash is inocul
This process is repeated until the starter eventually secured is suffici
large to seed the main mash. For further details, the reader is referr
the report submitted by Eoff, Linder, and Beyer to the Commission
Internal Revenue on May 6, 1918.[1]

Sodium carbonate in the form of soda ash is preferable for use i
fermentation on account of its comparatively low cost, although p
sium carbonate produces results that are just as favorable. Sodium
potassium hydroxides, sodium perborate, and other substances ma
used also to produce alkalinity. Not much more than 5 per cent of so
carbonate (calculated on the weight of the mash) should be use

---

[1] The facts contained in this report were reproduced in *Ind. Eng. Chem.*, 1
(1919).

vise the fermentation will be permanently inhibited. On the other the highest possible concentration (close to 5 per cent) of the nate should be employed, for the yield of glycerol is increased by sing the alkalinity of the mash almost to the limit of endurance yeast. The final amount of alkali in 100 ml. of mash may be equiv- to 95 ml. of normal sodium hydroxide.

soon as the fermentation becomes vigorous, sodium carbonate is as a solid. A precipitate forms after the addition of the carbonate, ne evolution of gas temporarily ceases. The precipitate disappears ally, and fermentation again proceeds. Thereafter sodium carbon- added in as large amounts and as rapidly as the yeast will tolerate. ly the alkali is added in accordance with a definite schedule. Eoff n favor of adding it in five portions: the first portion containing ximately 12.5 per cent of the total carbonate to be added; the second n, about 22 per cent; the third, about 31 per cent; the fourth, about cent; and the fifth, about 12.5 per cent.

ing the fermentation the temperature should be kept within the of 30 to 32°C. in order to ensure high yields.

n 20 to 25 per cent of the sugar of the mash is converted to glycerol, considerable quantities of ethyl alcohol and carbon dioxide are ed.

' and his associates obtained successful results from the fermenta- mashes of 2,000-gal. capacity.

cerol yields of 10.5 to 24.2 per cent (based on total fermentable ) and corresponding ethanol yields of 36 to 27 per cent were ed by fermentation of molasses in the presence of sodium carbonate esult of the use of 41 strains of wine and brewers' and distillers' (Freeman and Donald, 1957*c*). The highest yields of glycerol er cent) and ethanol (29 to 32 per cent) were obtained from pure s of a wine yeast and a strain of distillers' yeast.

yields of glycerol and acetic acid increased and that of ethanol ed with an increase in the dosage of sodium carbonate from 0 to cent (Freeman and Donald, 1957*c*).

**Fulmer-Underkofler-Hickey Process.** Fulmer, Underkofler, and (1947) have patented a process for producing glycerol in an acid n, in which ammonium sulfite and/or ammonium bisulfite are pres- excess. The pH is maintained at about 6.8 by means of a pH r-controller, using sulfur dioxide or sulfurous acid.

process is carried out as follows: A medium, of which the one bed below is typical, is prepared.

| Ingredient | Weight/Liter, g. |
|---|---|
| Sugar (as dextrose) | 150 |
| Corn-steep liquor (heavy) | 2.0–4.5 |
| $NH_4Cl$ | 1.5 |
| $K_2HPO_4 \cdot 3H_2O$ | 0.75 |
| $KH_2PO_4$ | 0.75 |
| $MgSO_4 \cdot 7H_2O$ | 0.2 |
| $CaCl_2$ | 0.1 |

Ammonium sulfite and/or ammonium bisulfite is used in an a
equivalent to 60 g. of $SO_2$. The pH of the medium is adjusted t
The medium is then inoculated with a culture of yeast acclimati
ammonium sulfite. An amount of inoculum representing 10 per c
the volume of the medium is satisfactory. After the fermentation be
active, a portion of the ammonium sulfite and/or bisulfite is add
5-g. portion may be added, at intervals of approximately 6 hr. The
may be added as a solid or as ammonia or ammonium hydroxide
bined with sulfur dioxide or sulfurous acid.

In recovering the glycerol, there is added a quantity of calcium hy
ide in excess of that required to make the medium alkaline. The vo
substances are removed by distillation, and the ammonia, acetalde
and ethanol are separated. The solids may be removed by centrif
or filtering, after which carbon dioxide is added to precipitate the ca
in solution. The precipitate of calcium carbonate is removed by ce
fuging or filtering. Glycerol remains in the residue.

In an earlier patent, Fulmer and his associates (1945) describ
process for producing glycerol in an acid medium containing magne
sulfite as the aldehyde-fixing agent. The pH was maintained at 6.5 w
pH recorder-controller, by using 50 per cent acetic acid. The ferme
tion was complete in 96 hr. Yields were obtained of 23.15 per cen
glycerol, based on the dextrose used.

**The Schade-Färber Process.** Schade and Färber (1947) and Sch
(1947) have patented processes for producing glycerol in which the v
tile constituents are removed from the fermentation medium by bubb
nitrogen, oxygen, or air through it. The principal volatile substances
ethanol, carbon dioxide, and acetaldehyde. They may be recovered
passage through special scrubbing towers.

In one process, glycerol is produced in the presence of magnesium
bonate. The pH is maintained between 7 and 8 by the addition o
to 20 parts of magnesium carbonate for each 100 parts of ferment
carbohydrate present initially in the medium. For example, 10 liter
medium (obtained by hydrolyzing wheat) containing 1,700 g. of redu
sugar were added, together with 170 g. of compressed yeast (of 72 per
moisture content), to a fermentation vat that was equipped with a stir
a gas disperser, and gas outlets. The vat was located in a room ma
tained at a constant temperature of 32°C. The medium was aerated
the rate of 1 liter per min. The pH was kept in the range 7.0 to 7.2 by
addition of a total of 170 g. of magnesium carbonate. Within 26
almost all of the fermentable sugar had been consumed. A yield
obtained of 310 g. of pure glycerol and 400 g. of yeast, which could
reused.

In a second process, the volatile products are removed as they are p
duced and the pH is maintained between 6.9 and 7.3 by the additio
neutralizing agents, such as hydroxides, alkaline-reacting phospha
carbonates of the alkali metals, etc. It is claimed that media contai
10 to 20 per cent of carbohydrates may be fermented in 12 to 24 hr. a
to 37°C. In one example, 10 liters of a solution containing 1,000 g

ing sugars were placed with 100 g. of compressed yeast of 72 per cent ure content in a vat equipped and aerated as described above. The as maintained at 6.9 to 7.3 by the addition of a 5 per cent solution lium hydroxide. Within 12 hr., 795 g. of the fermentable sugar had consumed. There were recovered 310 g. of yeast (72 per cent mois- and 150 g. of pure glycerol.

**ycerol from Molasses.** Duchenne (1942) revealed that glycerol was produced from molasses on an industrial scale in South Africa, an alkaline fermentation. He described the production of glycerol, calcium sulfite, and reported that a sugar factory that produced tons of molasses a year could manufacture 6 tons of glycerol and gal. of ethanol each day.

**ycerol from Hydrolyzed Starch-containing Materials.** Lees (1944) d the production of glycerol from starch-containing materials, using n or magnesium sulfite. Enzyme-saccharified starch-containing ials were unsatisfactory for glycerol production, since maltose was ief end product and maltose, even in pure form, was fermented very . Acid-hydrolyzed cornstarch or dry-milled corn products were actory for glycerol production. With magnesium sulfite, yields of 24 per cent glycerol (on the basis of dextrose) were obtained; with n sulfite, yields as high as 30 per cent were secured. However, it ecessary to use large inocula of yeast and high concentrations of n sulfite to obtain yields above 25 per cent.

ade and Färber, and Schade used hydrolyzed wheat and other successfully for glycerol production.

**eoretical.** According to Neuberg's scheme for the production of ol in an alkaline medium, 1 molecule of acetic acid is produced with molecules of glycerol:

$$2C_6H_{12}O_6 + H_2O \rightarrow CH_3COOH + 2C_3H_8O_3 + C_2H_5OH + 2CO_2$$

atio of glycerol to acetic acid is 184:60, or 3.07:1. Some actual varied from 2.91:1 to 3.12:1 (Lawrie, 1928).

**cerol from Synthetic Sugar.** Lüdecke (1938) has patented a proc- producing glycerol from the sugar or sugar sirup derived from the nsation of formaldehyde in an alkaline solution. (For details of the nsation process, consult German Patent 590236.)

**s of Glycerol.** Glycerol finds a wide variety of uses. These its use as a solvent; as a sweetening agent; as a constituent of ents, lotions, antiseptics, adhesives, and inks; as a food; and as an eeze agent. It is used in the preparation of biological media and lycerine (Lesser and Murphy, 1936). It may be used in the manu- e of synthetic rubber, glycerine-litharge cements, and model- ays. New uses for this trihydric alcohol are continually being

**her Information.** Additional information concerning the glycerol tation may be obtained by a study of the publications listed at the this chapter. Much valuable data will be found in the patent ure, especially that of Germany.

### References on Glycerol Fermentation and Glycerol

Duchenne, J. O.: Manufacture of Glycerin from Sugar by Fermentation, *Proc* *Ann. Congr. S. African Sugar Technol. Assoc.,* Apr. 21–23, 1942, pp. 45–47.

Eoff, J. R., W. V. Linder, and G. F. Beyer: Report to the Commissioner of In Revenue, U.S. Department of the Treasury, May 6, 1918.

———, ———, and ———: Production of Glycerin from Sugar by Ferment *Ind. Eng. Chem.,* **11**: 842 (1919).

Freeman, G. G., and G. M. S. Donald: Fermentation Processes Leading to Gly I. The Influence of Certain Variables on Glycerol Formation in the Presen Sulfites, *Appl. Microbiol.,* **5**(4): 197–210 (1957*a*).

——— and ———: II. Studies on the Effect of Sulfites on Viability, Growth Fermentation of *Saccharomyces cerevisiae, Appl. Microbiol.,* **5**: 211–215 (19

——— and ———: Studies on Glycerol Formation in the presence of Alkalies, *Microbiol.,* **5**: 216–220 (1957*c*).

Henneberg, W.: "Handbuch der Gärungsbakteriologie," 2d ed., Paul Parey, B 1926.

Lawrie, J. W.: "Glycerol and Glycols," Reinhold Publishing Corporation, Inc., York, 1928.

Lees, T. M.: The Fermentative Production of Glycerol, *Iowa State Coll. Jour.* **19**(1): 38–40 (1944).

Leffingwell, G.: Glycerine Facts, *Glycerine Producers' Assoc., Trade Paper* New York.

Lesser, M. A., and J. R. Murphy: Glycerine—Its Role in Medicine, *Am. Pro* *Pharmacist,* June, July, August, 1936.

May, O. E., and H. T. Herrick: Some Minor Industrial Fermentations, *Ind.* *Chem.,* **22**: 1172 (1930).

Miner, C. S., and N. N. Dalton: "Glycerol," Reinhold Publishing Corporation, New York, 1953.

Stephenson, M.: "Bacterial Metabolism," 3d ed., Longmans, Green & Co., New York, 1948.

Underkofler, L. A.: "Industrial Fermentations," Vol. I, Chap. 8, edited by L. Underkofler and R. J. Hickey, Chemical Publishing Company, Inc., New Y 1954.

### Some Patents

Aktieselkabet Dansk Gaerings-Industri, Danish Patent 62,582, Aug. 21, 1944.

Blair, J., W. A. Caldwell, and T. A. McLellan: Recovery of Glycerol, U.S. Pat 2,741,638, Apr. 10, 1956.

Carothers, W. H., J. W. Hill, and F. J. L. Van Natta: Method of Manufactur Fermentation Glycerol, U.S. Patent 1,936,497, Nov. 21, 1933.

Cocking, A. T., and C. H. Lilly: Production of Glycerine by Fermentation, Patent 1,425,838, Aug. 15, 1922. Also British Patent 164,034, 1921.

Connstein, W., and K. Lüdecke: Process for the Manufacture of Propantriol fr Sugar, U.S. Patent 1,511,754, Oct. 14, 1924. Also German Patents 2985 298596, 1915; 13860, 1917, etc.

Eoff, J. R.: Process of Manufacturing Glycerol, U.S. Patent 1,288,398, Dec. 17, 19

Frankel, E. M., and S. L. Goldheim: Glycerol Manufacture, U.S. Patent 2,772, Nov. 27, 1956.

Fulmer, E. I., L. A. Underkofler, and R. J. Hickey: Glycerol Production, Patent 2,338,840, Nov. 13, 1945.

———, ———, and ———: Fermentative Production of Glycerol, U.S. Pat 2,416,745, Mar. 4, 1947.

Haehn, H.: British Patent 488,464, July 7, 1938; also French Patent 829,263, June 1938.

Henkel & Cie G.m.b.H.: German Patent 664576, Aug. 29, 1938.

Hildebrandt, F. M., and N. M. Erb: Glycerol Fermentation Process, U.S. Pat 2,169,245, Aug. 15, 1939.

e, J. W.: Acetic Acid Manufacture from Glycerol Fermentation Residues, .S. Patent 1,909,183, May 16, 1933.

C. H.: Manufacture of Glycerine, U.S. Patent 1,987,260, Jan. 8, 1935.

ke, K.: Verfahren zur Gewinnung von Glycerin aus Kohlenhydraten durch irung, German Patent 658047, Apr. 27, 1938.

A. C., G. A. Ledingham, and A. C. Blackwood: Production of Glycerol by ermentation, U.S. Patent 2,432,032, Dec. 2, 1947.

eutsche Hefeindustrie A.-G.: German Patent 664575, Aug. 29, 1938.

, A. L.: Production of Glycerol and Yeast by Fermentation, U.S. Patent 428,766, Oct. 7, 1947.

and E. Färber: Process for the Manufacturing of Glycerin, U.S. Patent 414,838, Jan. 28, 1947.

sley, R. A.: Recovery of Glycerin from Fermented Liquors, U.S. Patent 422,453, June 17, 1947.

r, K.: Process for the Recovery of Glycerine, U.S. Patent 2,153,704, Apr. 11, 39.

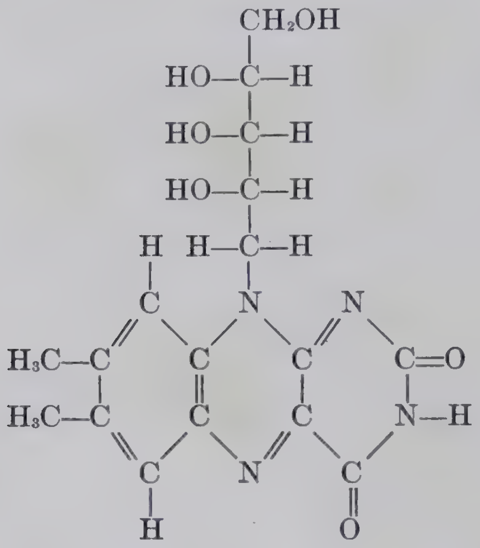
## CHAPTER 10

# VITAMIN PRODUCTION BY YEASTS AND YEAST-LIKE MICROORGANISMS

Comparatively large numbers of species of microorganisms have b shown to be able to synthesize vitamins of the B complex, but only a co paratively few are used to produce vitamins industrially.

### RIBOFLAVIN AND ITS PRODUCTION

Riboflavin (vitamin $B_2$, vitamin G, lactoflavin) has the empirical fo mula $C_{17}H_{20}N_4O_6$ and the structural formula indicated below:



When in the form of a crystalline yellow-orange powder, it is bitter i taste and practically odorless. It is hygroscopic and relatively stable t mineral acids and heat. However, it is sensitive to alkalies and is decomposed by ultraviolet and visible light radiations.

Riboflavin is essential for growth and reproduction in human being and animals. It has assumed large importance as a component of feed for poultry, swine, calves, sheep, dogs, horses, fox, mink, and othe animals.

**Microorganisms Producing.** Riboflavin may be produced by a number of microorganisms, including yeasts, yeast-like microbes, and bacteria. Table 64 lists some of the microbes which are capable of synthesizing this vitamin. The most important of these are *Eremotheciu ashbyii, Ashbya gossypii,* and certain *Clostridium* species.

TABLE 64. SOME MICROORGANISMS CAPABLE OF SYNTHESIZING RIBOFLAVIN

| Yeast-like | Yeasts | Bacteria |
|---|---|---|
| *ya gossypii* * | Anascosporogenous: | *Clostridium butyricum* * |
| *othecium ashbyii* * | *Candida arborea* † | *Cl. acetobutylicum* * |
| | *C. flareri* † | *Cl. felsineum* |
| | *C. guilliermondia* † | *Cl. propylbutylicum* |
| | *C. utilis* | *Cl. roseum* |
| | *Rhodotorula* sp. | *Aerobacter aerogenes* |
| | *Torulopsis sp.* | *A. cloacae* |
| | Ascosporogenous: | *A. oxytocum* |
| | *Hansenula suaveolens* | *Azotobacter agile* |
| | *Saccharomyces* sp. | *Az. chroococcum* |
| | | *Az. vinelandii* |

nployed industrially for production of riboflavin.
nployed on a pilot-plant scale.

**oduction by A. gossypii.** *A. gossypii* was isolated and described by ll (1917). It is the causative agent of a disease of cotton bolls ecially in South Africa), which produces rotting and staining. It may cause infection of beans, citrus fruit, coffee, okra, and tomatoes. use of its pathogenicity, it is essential to sterilize all fermentation ues and cultures before they are discarded.

e cultural characteristics and morphology of this organism have been ibed by Pridham, Hall, and Pfeifer (1950) and by others.

illiermond, Fontaine, and Raffy (1935) recognized the ability of *ssypii* to form riboflavin.

ch of the early research on riboflavin production by *A. gossypii* was d out by scientists of the Northern Utilization Research Branch of .S. Department of Agriculture. Reference is made in this connection e publications of Wickerham et al. (1946), Tanner et al. (1948, , Pridham et al. (1950), and Pfeifer et al. (1950).

*trients Required.* *A. gossypii* requires certain nutrients for growth naximum yields of riboflavin. Pridham and associates reported that ercial glucose appeared to be the best carbon source, but that se and maltose could be substituted for it. Starch, molasses (cane- , beet-sugar, and corn), and the common pentoses were apparently ilized by *A. gossypii* for synthesis of riboflavin. Peptone and animal- liquor were especially good sources of nitrogen, while corn-steep r was the most acceptable of the plant protein sources. Biotin, thia- and *meso*-inositol were required for adequate growth of the organism.

*ck Cultures.* *A. gossypii* may be transferred at weekly intervals on lium containing 0.5 per cent peptone, 0.3 per cent yeast extract, r cent malt extract, 1.0 per cent commercial glucose, and 2.0 per cent nd incubated in the temperature range of 27 to 30°C.

*culum Development.* Pfeifer et al. (1950) described a method for ring an inoculum for pilot-plant fermentations. A loopful of a 24-hr.

culture of *A. gossypii* NRRL Y-1056 was placed in 100 ml. of the fo
ing medium in a 500-ml. flask and incubated for about 24 hr. on a rec
cating shaker at 26 to 30°C.: 2 per cent glucose, 0.5 per cent pep
1 per cent corn-steep liquor, and sufficient water to make 100 ml. Th
of this medium before sterilization for 30 min. at 121°C. was 6.5.
contents of this flask were used to seed 6 liters of sterilized medium o
following composition contained in a 9-liter glass bottle: 2 per cent
cose, 1 per cent corn-steep liquor, 0.5 per cent animal-stick liquor
water to make 6 liters. The pH of this medium before sterilization
45 min. at 121°C. was 6.5. The organism was grown for 24 hr. with a
tion provided by passing sterile air through a perforated tube locate
the bottom of the bottle. This culture was used to inoculate 20
300 gal. of sterilized medium in a fermentor.

*Production.* According to Pfeifer and associates, satisfactory yiel
riboflavin may be obtained when fermentation variables are caref
controlled. They advocated the use of a medium which contained 2
cent glucose, 1.8 to 2.1 per cent suitable corn-steep liquor, 1 per cent anim
stick liquor, and a small amount of an antifoam agent. They rec
mended continuous sterilization of the medium at a pH of 4.5 and a
temperature of 275°F. (135°C.) for 5 min. An inoculum of 0.5 to 1
cent should be used for seeding fermentors. Sufficient aeration for a
quate mixing of the medium but not hampering growth should be p
vided. An aeration rate of about 0.25 volume of air per volume
medium per minute was satisfactory. Fermentation was carried out a
temperature of 83 to 85°F. (28.3 to 29.4°C.) for 96 to 120 hr. The yie
were 500 to 600 γ per ml. of riboflavin. The fermentation liquor thus p
duced may be evaporated to a sirup and then dried on drum driers
yield a concentrate containing 2.5 per cent of riboflavin.

A flow sheet for the process described by Pfeifer et al. is shown
Fig. 36.

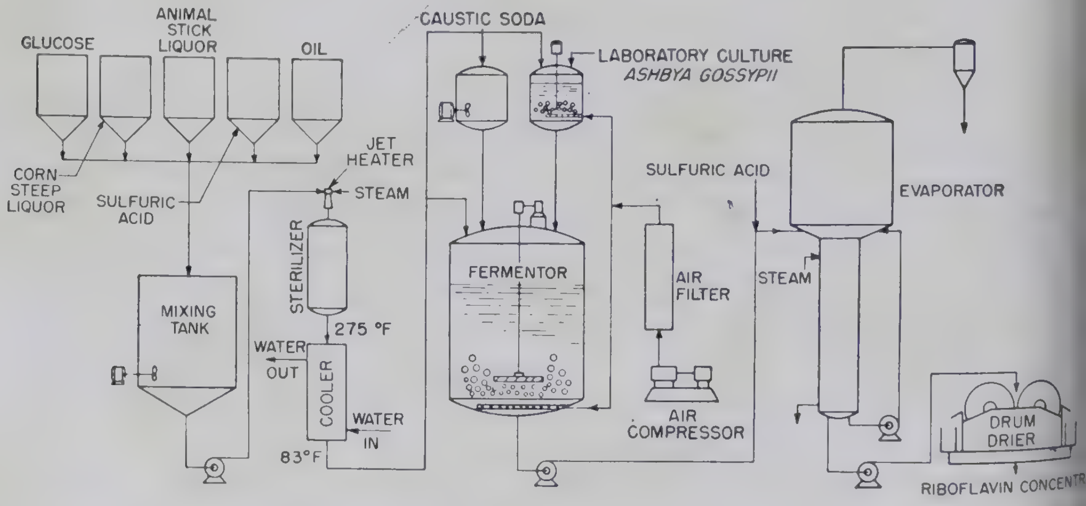

GLUCOSE
ANIMAL STICK LIQUOR
OIL
CORN-STEEP LIQUOR
SULFURIC ACID
JET HEATER
STEAM
MIXING TANK
STERILIZER
275 °F
WATER OUT
COOLER
WATER IN
83°F
CAUSTIC SODA
LABORATORY CULTURE
ASHBYA GOSSYPII
FERMENTOR
SULFURIC ACID
AIR FILTER
AIR COMPRESSOR
STEAM
EVAPORATOR
DRUM DRIER
RIBOFLAVIN CONCENTR

FIG. 36. Flow sheet for riboflavin production by fermentation. [*Courtesy of* V.
*Pfeifer, F. W. Tanner, Jr., C. Vojnovich, and D. H. Traufler: Riboflavin by Ferm
tation with* Ashbya gossypii, *Ind. Eng. Chem.*, 42: 1776 (1950). (*Copyright* 1950
*the American Chemical Society and reprinted by permission of the copyright own*

nedium containing 2 per cent of commercial glucose, 0.7 per cent of ne, 1 per cent of dried wheat-fermentation slop and sufficient cobalt e to furnish 10 p.p.m. of cobalt was used by Hickey (1954) for ribo- production. The pH of the medium was adjusted to 6.8. A yield 5 γ per ml. was obtained after fermentation for 5 days at 28°C. with ion and agitation.

iley et al. (1951) reported on the biosynthesis of riboflavin and other h factors by *A. gossypii* NRRL Y-1056 in grain-stillage media on atory and pilot-plant scales. They used a medium containing 50 per stillage, 1 per cent packing-house-stick liquor, and 2 per cent com- al glucose for experiments in 1,000-gal. pilot-plant fermentors. It ound that higher yields of riboflavin were obtained when the medium lash-sterilized at 280 to 290°F. (137.8 to 147.3°C.) for 3 min. than batch-sterilized at 250°F. (121°C.) for 1 hr. ✓

**oduction by E. ashbyii.** Riboflavin is produced industrially from *hbyii*, a yeast-like organism belonging to the Ascomycetes. The was described by Guilliermond (1935), who together with Fontaine Raffy (1935), noted that it produced a yellow pigment related to avin group. Mirimanoff and Raffy (1938) extracted the crystalline from *E. ashbyii*. It was found by Schopfer (1944) that *E. ashbyii* red biotin, inositol, thiamin, and some constituents of peptone for h and that riboflavin production was stimulated by peptone.

oflavin production has been described in patents. In one method bed by Rudert (1945), it is produced by *E. ashbyii* from substan- carbohydrate-free media. Based on the total weight of nutrients, nedium contains 10 to 90 per cent of proteinaceous material, a olizable lipid, and nutrients such as peptone or a combination of salts per cent $KH_2PO_4$, 0.07 per cent $MgSO_4 \cdot 7H_2O$, 0.10 per cent NaCl. 001 per cent $FeSO_4 \cdot 7H_2O$). Examples of the media and lipids used, elds obtained, are shown in Table 65.

TABLE 65. RIBOFLAVIN PRODUCTION FROM DIFFERENT MEDIA *

| ource | G./100 ml. | Peptone, 2.4 g./100 ml. (μg./ml.) | Salts and egg albumen, 0.6 g./100 ml. (μg./ml.) | Initial pH | Final pH |
|---|---|---|---|---|---|
| il | 0.6 | . . . | 178 | 6.5 | 7.1 |
| oil | 0.6 | . . . | 113 | 6.5 | |
| oil | 0.6 | 110 | . . . | 6.5 | 7.1 |
| oil | 0.6 | . . . | 107 | 6.5 | 6.7 |
| oil | 0.6 | 134 | . . . | 6.5 | 7.1 |

. Rudert, U.S. Patent 2,374,503, Apr. 24, 1945.

arrying out production, the following procedure is illustrative of 's invention: The media are adjusted to an initial pH of 5.5 to 7.5 spensed in containers to give a depth of 0.5 in., sterilized at 20 lb.

pressure for 45 min., cooled to 30°C., and inoculated with 0.7 per
of an active culture of *E. ashbyii.* During production the temperatu
maintained between 20 and 34°C. and the medium is aerated with 1
2 cu. ft. of sterile air per minute per square foot of mash surface. A
end of 50 to 90 hr. the conversion is complete and the final pH is us
6.5 to 7.2. The dried residues contain 200 to 6,000 μg. per g. of ribofl

In a patented method, described by Piersma (1946), *E. ashby*
grown on a medium containing animal proteinaceous material, a ca
hydrate and malt extract. The proteinaceous material may be l
pancreas, spleen, lung, or other substance of related nature.
carbohydrate source may be glucose, maltose, sucrose, molasses,
sirup, etc., or a combination of several of these. The concentra
employed may be 1 to 5 per cent proteinaceous material, 0.25 to 1.5
cent carbohydrate, and 0.5 to 5 per cent malt extract (optimum 1.
2.0 per cent). The pH of the medium is initially 5.5, and the tempera
27 to 30°C. Oxygen is supplied by aeration. The yield of riboflavi
generally 150 to 500 γ per ml. of medium; for example, in a mash cont
ing 4 per cent liver cake (residue from aqueous extract of liver), 0.5
cent sucrose, and 1.75 per cent malt extract, the yield of riboflavin
304 γ per ml. in 88 hr.

Gaden, Petsiavas, and Winoker (1954) reported on the productio
riboflavin from citrus molasses, using *E. ashbyii* NRRL 1363. The ci
molasses was clarified by settling followed by decantation. The inocu
was prepared by growing the organism for 24 hr. in a sterilized med
containing 1.5 per cent clarified citrus molasses, 0.3 per cent yeast extr
(Difco), and 1.0 per cent peptone, which was adjusted to pH 6.6 to
before sterilization. The authors recommended the use of an initial c
centration of citrus molasses equivalent to approximately 6.0 per c
reducing sugar, fortified by the addition of a commercial enzym
yeast hydrolyzate (Basamin-Busch) in a concentration of 0.3 per cent.
the production of the highest yields of riboflavin. The pH of the proc
tion medium was adjusted to 6.5 to 8 before sterilization, which gener
resulted in a pH of about 6.4 to 6.8 after sterilization. This product
medium was inoculated with 4 per cent by volume of inoculum. Yi
of as much as 720 μg. per ml. of riboflavin were obtained in 7 to 9 d
at 27 to 30°C. in shake flasks.

Table 66 lists various raw materials which have been used for ribofla
production by *E. ashbyii.*

**Production by Candida Species.** The production of riboflavin
species of the genus *Candida* has been studied by Burkholder (19
1944); Tanner, Vojnovich, and Van Lanen (1945); Tanner and
Lanen (1947); and others.

Burkholder (1943) found that *C. güilliermondia* grew and produ
riboflavin satisfactorily in media containing dextrose, mannose, levul
or sucrose. Asparagine and glycine were suitable and relatively inexp
sive sources of nitrogen for riboflavin production.

Burkholder (1944) patented a process for producing riboflavin f

BLE 66. SOME RAW MATERIALS SUGGESTED FOR USE IN PRODUCING RIBOFLAVIN BY *Eremothecium ashbyii* *

| rbon sources | Nitrogen sources | | Vitamins |
|---|---|---|---|
| ohydrates:<br>lactose<br>ucose<br>ycerol<br>ctose<br>altose<br>annose<br>ffinose<br>crose<br>ds:<br>itterfat<br>coa butter<br>conut oil<br>d-liver oil<br>rn oil<br>ttonseed oil<br>eam<br>rd oil<br>cithin<br>argarines<br>enhaden oil<br>ive oil<br>anut oil<br>ortenings | Albumen (egg)<br>Beans<br>Blood and serum (dried)<br>Bone meal<br>Brain-heart infusion<br>Brans (corn, rice, wheat)<br>Casein<br>Corn germ<br>Corn gluten<br>Corn-steep liquor<br>Cottonseed meal<br>Distillers' solubles<br>Fish meal<br>Hide scraps | Lentils<br>Linseed meal<br>Liver cake<br>Malt extract<br>Meat scraps<br>Oat hulls<br>Peas<br>Peptone<br>Skim milk<br>Spleen<br>Soybean meal<br>Stillage<br>Wheat<br>Wheat germ<br>Yeast<br>Yeast extract | Biotin<br>*Meso*-inositol<br>Thiamin |

ompiled from data published by T. G. Pridham in *Econ. Botany*, **6**(2): 185–205 2).

*uilliermondia* (A.T.C.C. 9058). A medium of the following composi- proved to be "highly satisfactory":

| | G./Liter | | Parts/ Million |
|---|---|---|---|
| $PO_4$ | 0.5 | Boron * | 0.01 |
| $O_4 \cdot 7H_2O$ | 0.5 | Manganese * | 0.01 |
| $_2 \cdot 2H_2O$ | 0.3 | Zinc * | 0.07 |
| $_4)_2SO_4$ | 2.0 | Copper * | 0.01 |
| | 0.1 | Molybdenum * | 0.01 |
| ragine | 2.0 | Iron * | 0.01 |
| trose | 20.0 | Biotin † | 1 μg. |

Supplied as chlorides or soluble salts.
Supplied as methyl ester or crude concentrate.

lost suitable results were obtained when the pH was adjusted to 5 , although the range of 5 to 7 was satisfactory. The temperature 30°C. Increased yields of riboflavin were obtained by adding small unts of sterile potassium cyanide or cyanide gas to the medium after vigorous fermentation was obtained, usually after 24 hr. The fer tion time was 6 to 7 days.

Tanner and Van Lanen (1947) have patented a method for pro riboflavin from *C. flareri.* The method briefly consists of growing aerobic conditions at 30°C. for about 7 days *C. flareri,* or other s *Candida* species, in a medium containing a fermentable sugar, an as ble source of nitrogen, noniron inorganic salts, biotin, and les 10.3 μg. of iron per 100 ml.

The preferred species of *Candida* are *C. flareri* and *C. guillierm* but other suitable species may be used.

It is essential to use great care in controlling the iron content medium, for iron exerts a critical influence on the yield. A low iro tent may be obtained in several ways, among which are the follo (1) by the treatment of the medium by its passage through a exchange apparatus for the removal of iron (for example, with the Nalcite MX, Nalcite iron remover, or Nalcite AX); (2) by the sel of the ingredients of known low iron content; or (3) by the treatm the medium with chloroform and 8-hydroxyquinoline. In the latter cedure, the medium (about 200 ml. of the basal) is shaken in a separ funnel with a mixture of 10 mg. of 8-hydroxyquinoline in about of chloroform. The mixture is allowed to stand until the chlor separates out, after which the latter is withdrawn. Additional ch form is added, the mixture shaken and allowed to settle, and the chloroform separated out. This procedure is repeated, 8-hydroxyq line being added in alternative extractions, until the chloroform that settles out appears to be colorless, which indicates that not than 0.3 μg. of iron per 100 ml. remains in the medium.

Various media may be used as long as they conform to the ge specifications outlined above and contain less than 10.3 μg. of iro 100 ml. Tanner and Van Lanen, in several experiments, used a medium that contained, dissolved in 200 ml. of water, 40 g. of glu 2 g. of asparagine, 2 g. of urea, 0.5 g. of $KH_2PO_4$, 0.5 g. of $MgSO_4 \cdot 7$ and 1 μg. of biotin (free acid). This basal medium was treated chloroform and 8-hydroxyquinoline to remove most of the iron and up to 1 liter with triply distilled water in a container that woul contribute iron. Portions of 100 ml. of the medium were dispens 500-ml. Erlenmeyer flasks, sterilized at 126°C. for 15 min., cooled inoculated with a suspension of yeast cells which had been washed p ously with triply distilled water in a centrifuge to remove loosely a ent iron.

As an alternate procedure, the glucose is dissolved in 1 liter of and then treated by passage through an ion-exchange column conta Nalcite MX (or other suitable iron remover). The other ingred mentioned above are then added, and the pH is adjusted to 5 (4.5 to with NaOH or other alkali. The medium is sterilized, cooled, and in lated as described above.

Tanner and Van Lanen state in their patent that various substitu may be made in the basal medium. Fermentable sugars, such as a

·, dextrose, levulose, maltose, mannitol, sucrose, or xylose may be
l. Ammonium chloride, ammonium nitrate, ammonium sulfate, or
ionium phosphate may be employed as the source of inorganic
ogen, while asparagine, glutamic acid, or hydrolyzed casein may be
loyed as the source of organic nitrogen. Phosphorus may be supplied
hosphoric acid, dipotassium hydrogen phosphate, or ammonium phos-
te; sulfur, as magnesium sulfate, sodium sulfate, or potassium sulfate;
ssium, as dipotassium hydrogen phosphate or potassium sulfate; and
nesium, as magnesium sulfate, magnesium chloride, or magnesium
ate. Biotin may be supplied purified as the free acid or the methyl
r, or in its natural forms.

gitation and aeration increase the yields and shorten the fermenta-
time.

laximum yields were obtained from the basal medium described
ve when the iron concentration was between 0.5 and 1.3 μg. per
ml. Table 67 indicates the influence of the iron concentration on the
d at the end of 7 days.

TABLE 67. EFFECT OF IRON CONCENTRATION ON RIBOFLAVIN PRODUCTION BY SELECTED SPECIES OF *Candida* *

| Culture | Iron concentration, μg./100 ml. | Dry wt. of yeast, g./100 ml. | Dry wt. of cell-free residue, g./100 ml. | Riboflavin, μg./ml. |
|---|---|---|---|---|
| *lida guilliermondia* RRL 488) | 0.0– 0.3 | 0.27 | 0.45 | 108.0 |
| | 0.5– 0.8 | 0.57 | 0.67 | 123.0 |
| | 1.0– 1.3 | 0.66 | 0.56 | 120.0 |
| | 10.0–10.3 | 0.89 | 0.26 | 7.2 |
| | 50.0–50.3 | 0.92 | 0.19 | 3.2 |
| *uilliermondia* RRL 324) | 0.0– 0.3 | 0.21 | 0.37 | 107.0 |
| | 0.5– 0.8 | 0.67 | 0.33 | 125.0 |
| | 1.0– 1.3 | 0.61 | 0.33 | 157.0 |
| | 10.0–10.3 | 0.89 | 0.35 | 16.5 |
| | 50.0–50.3 | 0.82 | 0.19 | 10.6 |
| *areri* RRL 245) | 0.0– 0.3 | 0.42 | 1.30 | 195.0 |
| | 0.5– 0.8 | 0.49 | 0.75 | 216.0 |
| | 1.0– 1.3 | 0.55 | 0.72 | 216.0 |
| | 10.0–10.3 | 1.12 | 0.28 | 8.9 |
| | 50.0–50.3 | 1.31 | 0.52 | 4.1 |

F. W. Tanner, Jr., and J. M. Van Lanen, U.S. Patent 2,424,003, July 15, 1947.

**roduction by Lactose-fermenting Yeasts.** Rogosa (1942) has shown
lactose-fermenting yeasts have the ability to synthesize riboflavin
n grown on a riboflavin-free medium. The results of some of their
ings are summarized in Table 68.

**ecovery of Riboflavin.** Riboflavin may be recovered from produc-
substrates by a variety of procedures, many of them patented.

Table 68. Quantity of Riboflavin Synthesized by Some Lactose-fermenting Yeasts *

| Yeast | Ribofla Synthesi $\gamma$/ml. of C |
|---|---|
| *Saccharomyces anamensis* No. 145 | 0.08 |
| Type F No. 93 | 0.08 |
| *Candida* (*Monilia*) *pseudotropicalis* Castellani No. 32 | 0.06 |
| *C. pseudotropicalis* (*Mycotorula lactis*) No. 130 | 0.08 |
| *S. lactis* No. 131 | 0.09 |
| *C. pseudotropicalis* (*Torulopsis kefir*) No. 149 | 0.10 |
| *S. lactis* (*Zygosaccharomyces lactis*) No. 90 | 0.06 |
| *S. lactis* (*Z. lactis*) No. 27 | 0.10 |
| *Torulopsis sphaerica* (*Torula sphaerica*) No. 13 | 0.09 |
| *C. pseudotropicalis* (*T. lactosa*) No. 168 | 0.11 |
| *C. pseudotropicalis* (*T. cremoris*) No. 2 | 0.06 |
| *S. fragilis* No. 15 | 0.09 |

* M. Rogosa, *Jour. Bact.*, **45**(5): 459–460 (1942).

Keresztesy (1944) patented a procedure for extracting riboflavin butanol, followed by the use of other solvents, such as petroleum e and acetone. McMillan (1945) patented a chemical precipitation me in which a soluble reducing agent and a finely divided diatomaceous e were used. Hines (1945*a*) described a method wherein riboflavin adsorbed on fuller's earth, silica jel, or other adsorbent, and eluted an aldehyde, ketone, or alcoholic solution of an organic base. Ano procedure by Hines (1945*b*) related to the conversion of riboflavin less soluble form by the action of reducing bacteria, such as *Streptoco faecalis.* Dale (1947) has patented a method for securing crysta riboflavin from the precipitates produced by the reduction of this vita to a less soluble form by either reducing bacteria or chemical redu agents.

## SOME FACTORS AFFECTING VITAMIN PRODUCTION BY FOOD YEASTS

The vitamin content of yeast is influenced by the characteristics o yeast, the composition of the propagation medium, aeration, storage, other factors.

The thiamin (vitamin $B_1$) content of yeast may be increased number of ways. Growth in a medium rich in the vitamin or contai the thiazole and pyrimidine portions of the vitamin increases the thia (Van Lanen et al., 1942). Holding the yeast cream at 32 to 37°F. several days increases the thiamin content, according to Maisel (19 Brewers' yeast produced under fermentation conditions is often a be source of the vitamin than bakers' yeast propagated under stro aerobic conditions. However, when these two yeasts are cultivated u similar conditions and in the same medium, they usually contain a the same amount of thiamin. The thiamin contents of *Saccharom* visiae, *Candida utilis*, and *C. arborea* decreased with increasing ds of cells (Singh, Agarwal, and Peterson, 1948).

icotinic acid is formed in the largest amount of any of the vitamins he B complex by yeasts. This vitamin, like thiamin, is readily rbed from the medium. A two- to sixfold increase in the content of yeast cell may result from such additions (Van Lanen, 1947). Increas- the aeration of the medium increases the yield of cells from *S. cere-* *ae*, *C. utilis*, and *C. arborea* and the amount of nicotinic acid (Singh l., 1948).

iotin is also absorbed from the medium by some species of yeasts. It been demonstrated that the biotin uptake of *C. utilis* may be increased ld; that of *C. arborea*, up to 30-fold; and that of *S. cerevisiae*, about -fold when the vitamin is added to the medium (Singh et al., 1948). ng and Peterson (1949) reported that the uptake of biotin from the ium was not correlated with the requirement of the yeast for it. For nple, *Hansenula anomala* and *C. utilis* synthesize the biotin required growth and absorb very little of it from the medium.

he amount of *p*-aminobenzoic acid (PABA) produced by different ies of yeasts varies, according to the strain, the composition of the lium, aeration, and other factors. Agarwala and Peterson (1950*a*) nd that high yields of the vitamin were produced by *S. cerevisiae* and (*Mycotorula*) *lipolytica* and low yields by *C. utilis*, *C. krusoides*, *H.* *mala*, and *Rhodotorula glutinis* (*gracilis*). The last-named four yeasts thesized larger amounts of PABA from a xylose medium than from a ose medium and retained more in the cells. Agarwala and Peterson 50*b*) noted that the total synthesis of PABA per liter of medium was ter under aerobic conditions than under anaerobic conditions and that ppeared to be related to the yield of yeast.

he addition of pantothenic acid to the medium exerts no significant ct on the amount produced by *S. cerevisiae*.

yridoxin is absorbed from the medium by *S. cerevisiae* (Holden et al., 9).

iboflavin production is not increased significantly by its addition to medium. Comparatively large yields may be obtained by careful ction of the yeast and medium.

he synthesis of certain vitamins of the B complex appears to be ted to the iron content of the medium in the case of *C. utilis*. Lewis 44) found that a deficiency of iron in the production medium resulted increased rates of synthesis of thiamin, riboflavin, nicotinic acid, idoxin, and pyridoxin isotels and decreased rates of synthesis of tin, PABA, and inositol. No effect on the synthesis of pantothenic acid observed.

**Iethionine and Cystine Contents of Yeasts.** The methionine and cystine contents of a number of different species of yeasts are shown able 69. It will be noted that *R. glutinis*, a fat-producing yeast, con- ned the largest amount of methionine.

hiao and Peterson (1953) studied some factors relating to the rovement in yields of protein and methionine in yeasts. They found

TABLE 69. METHIONINE AND CYSTINE IN YEASTS GROWN ON HAWAIIAN-MOLASSES MEDIUM *

| Yeast | Yield, sugar fermented, % | Methionine of dry yeast, % | Cy o ye |
|---|---|---|---|
| *Candida krusoides* | 80.0 | 0.66 | 0 |
| *C. guilliermondia* | 67.2 | 0.74 | 0 |
| *C. pulcherrima* | 54.7 | 0.58 | 0 |
| *C. zeylanoides* | 69.0 | 0.82 | 0 |
| *Debaryomyces matruchoti* | 83.4 | 0.42 | 0. |
| *Endomycopsis fibuliger* | 63.8 | 0.17 | 0. |
| *E. magnusii* | 69.1 | 0.58 | 0. |
| *Hansenula anomala* | 72.0 | 0.50 | 0. |
| *H. saturnus* | 59.6 | 0.61 | 0. |
| *C. mycoderma (M. cerevisiae)* | 75.9 | 0.83 | 0. |
| *Geotrichum candidum (Oïdium lactis)* | 54.0 | 0.30 | 0. |
| *Pichia alcoholophila* | 66.5 | 0.48 | 0. |
| *Rhodotorula glutinis (gracilis)* | 73.3 | 1.00 | 0. |
| *Saccharomyces cerevisiae* Y-30 | 66.2 | 0.75 | 0.3 |
| *S. cerevisiae* 53 | 63.8 | 0.71 | 0.3 |
| *S. cerevisiae* 49 | 64.1 | 0.67 | 0.2 |
| *S. cerevisiae* G.M. | 63.5 | 0.78 | 0.2 |
| *S. cerevisiae* F-53 | 67.7 | 0.85 | 0.3 |
| *S. cerevisiae* F-22 | 60.1 | 0.73 | 0.2 |
| *C. utilis* 3 | 68.3 | 0.54 | 0.2 |

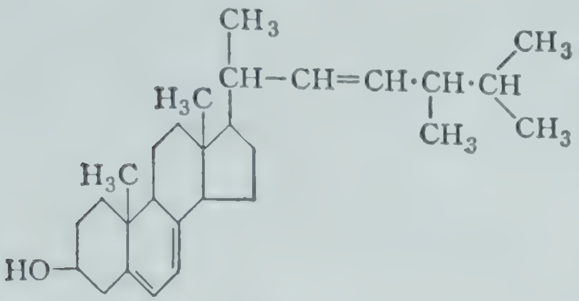
* J. S. Chiao and W. H. Peterson, *Jour. Agr. Food Chem.*, **1**(16): 1005 ( Copyright 1953 by the American Chemical Society and reprinted by permissi the copyright owner.

that increasing the inorganic nitrogen content of a beet-molasses me through the additions of ammonium sulfate and ammonium dihydr phosphate to 0.044 per cent increased the yields of cells and the nitr and methionine contents of *S. cerevisiae* F-53; however, additions of t salts to 0.089 per cent nitrogen resulted in a decreased yield of cells methionine but an increase in the nitrogen of the cells.

The addition of cystine, threonine, and choline to the synthetic me of Olson and Johnson (1949) did not increase the methionine conte *S. cerevisiae* F-53. Cystine increased the yield of cells greatly, howe

## ERGOSTEROL

Ergosterol ($C_{28}H_{44}O$) was discovered by Tanret (1889) in the erg rye (caused by *Claviceps purpurea*). It has the structure indicated be According to Bergmann (1953), it is the most widely distributed of $\Delta^{5,7}$-sterols. It is the chief sterol of fungi, lichens, and at least som the algae. It does not always occur in the free state and may be fo as the ester, especially ergosteryl palmitate. It may also be associ with other sterols.



**roduction by Molds.** A number of scientists have reported on the luction of sterols by molds. Birkinshaw et al. (1931) studied the iso- n and characterization of the ergosterol produced by *Penicillium rulum*, while Oxford and Raistrick (1933) investigated the ergosteryl iitate of *P. brevi-compactum* and related molds. Pruess and associ- (1931, 1932, 1934) studied the ergosterol content of species of *Asper- s*, *Penicillium*, and other molds. Pruess, Eichinger, and Peterson 4) found that the sterol contents of 24 molds studied by them ranged 0.26 to 1.70 per cent of the weight of the dried mycelium when the ls were grown on a glucose–malt-sprouts medium (refer to page 657). ck and coworkers (1935*a*, *b*) studied the factors influencing the th and sterol production by *Aspergillus fischeri.* Additional studies been carried out by Bernhauer and Patzelt (1935); Vanghelovici Serban (1940, 1941); Cavallito (1944); Zook et al. (1944); Savard Grant (1946); Angeletti and Tappi (1947); Ottke et al. (1950, 1951); er, Payne, and Appleton (1955); Appleton, Kieber, and Payne 55); and others.

ppleton and associates (1955) found that *P. westlingii* and *Neurospora a* formed the largest amounts of ergosterol (2.20 per cent and 1.07 ent, respectively, in the dried mycelia) of the 23 molds tested by i.

e used mycelium from industrial fermentations is a potential source erols.

**oduction by Yeasts.** Ergosterol production by yeasts has been ed by Bills et al. (1930); Prickett et al. (1930); Massengale et al. 1); Bennett (1936, 1942); Ramaswamy and Sreenivasaya (1946); dt and Foster (1954); Dulaney et al. (1954); Appleton, Kieber, and e (1955); and others.

ulaney, Stapley, and Simpf (1954) reported on an extensive study erning ergosterol production by a large number of cultures of yeasts. e of the details of this significant research will be given below.

ulaney and associates used a near-synthetic medium and a molasses– -steep medium for their survey. The near-synthetic medium, which used to survey 146 cultures of yeasts, contained 50 g. of glucose ilized separately), 5 g. of $(NH_4)_2HPO_4$, 1 g. of asparagine, 1 g. of m citrate, 1 g. of $MgSO_4 \cdot 7H_2O$, 1 g. of N-Z-amine (an enzymatic t of casein), 1 g. of yeast extract, 0.2 g. of $MnSO_4$, 0.005 g. of $_4 \cdot 7H_2O$, 0.005 g. of $CuSO_4 \cdot 5H_2O$, 0.005 g. of $ZnSO_4 \cdot 7H_2O$, and ient distilled water to make 1 liter. The optimum intial pH for this medium was found to be 7. Some results obtained with this mediu shown in Table 70. The molasses–corn-steep medium contained in blackstrap molasses at a concentration equivalent to 10 per cent of 2 per cent of corn-steep liquor, and distilled water to volume. T

TABLE 70. COMPARATIVE TEST OF *Saccharomyces cerevisiae* STRAINS IN TH NEAR-SYNTHETIC MEDIUM *

| Strain no. | After 7 days' growth | | After 9 days' growth | |
|---|---|---|---|---|
| | Dry-cell wt., mg./100 ml. | Ergosterol, per cent | Dry-cell wt., mg./100 ml. | Ergost per ce |
| MY14 | 985 | 2.0 | 1,109 | 3 |
| MY210 | 540 | 0.4 | 371 | 0.2 |
| MY212 | 1,363 | 0.2 | 739 | 0.4 |
| MY213 | 283 | 0.09 | 263 | 0.9 |
| MY215 | 318 | 0 | 290 | 1.1 |
| MY218 | 1,071 | 0.2 | 1,043 | 0.5 |
| MY223 | 240 | 0 | 235 | 1.3 |
| MY228 | 434 | 0 | 371 | 0.8 |
| MY232 | 301 | 0.1 | 620 | 1.2 |
| MY235 | 287 | 0.2 | 161 | 0.7 |
| MY238 | 1,180 | 0.4 | 1,214 | 0.5 |
| MY240 | 929 | 0.1 | 878 | 0.2 |
| MY241 | 648 | 0.1 | 569 | 0.3 |
| MY251 | 977 | 0.2 | 966 | 0.7 |
| MY256 | 242 | 0.4 | 216 | 1.1 |
| MY260 | 277 | 0.7 | 234 | 2.6 |
| MY266 | 1,185 | 0.5 | 1,220 | 0.7 |
| MY271 | 210 | 1.7 | 2,001 | 1.9 |
| MY274 | 254 | 4.0 | 235 | 4.9 |
| MY304 | 264 | 3.0 | 292 | 4.1 |
| MY306 | 397 | 8.5 | 389 | 9.8 |

* Courtesy of E. L. Dulaney, E. O. Stapley, and K. Simpf, *Appl. Micr* 2(6): 371 (1954).

of the medium was adjusted to 9 before sterilization and was 7 sterilization. The corn-steep liquor stimulates growth of the yeast

Special procedures were followed in building up the inocula fo foregoing survey media. The yeasts were grown on agar slants, w contained 1 per cent glucose, 1 per cent yeast extract, 2 per cent and sufficient distilled water to make up the desired volume, for 4 days at 28°C. The inoculum for the near-synthetic medium was 1 m a water suspension of the cells from an agar slant for each 50 m medium contained in a 250-ml. Erlenmeyer flask.

Two different types of media were used to prepare inocula fo molasses–corn-steep medium, which was used to survey most of the y cultures for ergosterol production. One of these contained 5 per ce glucose, 1 per cent of yeast extract, 1 per cent of N-Z-amine, 0.1 per of $KH_2PO_4$, and distilled water to volume. The other medium cont

TABLE 71. COMPARATIVE TEST OF NINE STRAINS OF *Saccharomyces cerevisiae* FOR ERGOSTEROL PRODUCTION IN THE MOLASSES–CORN-STEEP MEDIUM WITH SLURRY INOCULA *

| Strain no. | Inoculum | | | 1 day | | | 2 days | | | 3 days | | | 4 days | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Dry wt., mg./ 100 ml. | Sterol, mg./ 100 ml. | Sterol, per cent | Dry wt., mg./ 100 ml. | Sterol, mg./ 100 ml. | Sterol, per cent | Dry wt., mg./ 100 ml. | Sterol, mg./ 100 ml. | Sterol, per cent | Dry wt., mg./ 100 ml. | Sterol, mg./ 100 ml. | Sterol, per cent | Dry wt., mg./ 100 ml. | Sterol, mg./ 100 ml. | Sterol, per cent |
| MY14† | 1,250 | 45.6 | 3.65 | 1,928 | 46.4 | 2.41 | 2,089 | 76.4 | 3.66 | 2,060 | 86 | 4.17 | 1,951 | 73.4 | 3.76 |
| MY306 | 1,451 | 103 | 7.6 | 3,144 | 144 | 4.5 | 3,060 | 193 | 6.3 | 3,064 | 315 | 10 | 3,251 | 294 | 9 |
| MY784 | 1,332 | 123 | 9.2 | 3,073 | 132 | 4.3 | 3,123 | 204 | 6.4 | 3,225 | 261 | 8 | 3,319 | 267 | 8 |
| MY797 | 1,331 | 90 | 6.7 | 3,027 | 134 | 4.4 | 3,112 | 183 | 5.8 | 3,232 | 267 | 8.3 | 3,372 | 257 | 7.63 |
| MY798 | 1,099 | 112 | 10.1 | 2,733 | 200 | 7.2 | 2,840 | 256 | 9.0 | 2,778 | 299 | 10.7 | 2,996 | 317 | 10.6 |
| MY813 | 1,224 | 83 | 6.8 | 2,894 | 226 | 7.8 | 3,148 | 191 | 6.0 | 3,405 | 233 | 6.8 | 3,605 | 376 | 10.4 |
| MY814 | 1,043 | 74 | 7.1 | 2,695 | 114 | 4.2 | 2,872 | 120 | 4.3 | 2,973 | 195 | 6.5 | 3,950 | 231 | 5.8 |
| MY815 | 1,488 | 109 | 7.3 | 3,059 | 230 | 7.5 | 3,191 | 217 | 6.2 | 3,411 | 221 | 6.5 | 3,650 | 261 | 7.0 |
| MY816 | 1,300 | 111 | 8.6 | 2,800 | 159 | 5.6 | 2,780 | 106 | 3.8 | 2,987 | 158 | 5.3 | 3,118 | 278 | 8.9 |

* Courtesy of E. L. Dulaney, E. O. Stapley, and K. Simpf, *Appl. Microbiol.*, 2(6): 371 (1954).

† MY14 inoculum was grown in molasses–corn-steep medium for 7 days. The inoculum for each of the other yeasts was grown for 3 days in glucose 5 per cent, yeast extract 1 per cent, N-Z-amine 1 per cent, $KH_2PO_4$ 0.1 per cent, and distilled water to 100 per cent.

inverted blackstrap molasses and corn-steep liquor in the same pr tions used in the survey medium described above. The media we equal value, according to Dulaney and associates. A 50-ml. porti the inoculum medium (in a 250-ml. Erlenmeyer flask) was seeded 1 ml. of a water suspension of yeast cells grown for 4 to 7 days o agar medium described above. The flasks were incubated for 3 da rotary shakers at 28°C. Then they were placed on a bench top, an yeast cells were allowed to settle out (this usually required 1 to 2 The spent medium was decanted off, and 50 ml. of fresh molasses– steep medium was placed in each flask aseptically. The flasks reincubated at 28°C. on rotary shakers for 1 to 4 days. Assays were made to determine the dry weight of the cells and the sterol con Ergosterol was assayed by the procedure developed by Stoudt and F (1954).

As a result of testing 558 cultures of yeasts, representing 69 species 20 genera (and including 22 unidentified cultures), for their abilit produce ergosterol, Dulaney, Stapley, and Simpf concluded that amount of ergosterol produced in all species of yeasts, except for the g *Saccharomyces,* was extremely low (generally less than 0.1 per cent of weight of the dried cells); that *S. carlsbergensis, S. oviformis,* an *cerevisiae* were the best producers of ergosterol in the genus *Sacch myces;* that *S. cerevisiae* was the most promising species; that brew yeasts (strains of *S. cerevisiae*) were the best producers; and that yeasts appeared to be superior to all other strains.

Cell weights greater than 30 g. per liter were obtained from the strains of *S. cerevisiae,* while the ergosterol content ranged from 7 to per cent. Yields of ergosterol of between 3 and 4 g. per liter of medi were obtained under optimum conditions (refer to Table 71).

Appleton, Kieber, and Payne (1955) confirmed the conclusions Dulaney et al. (1954) that yeast strains of the genus *Saccharomyces* w the best producers of ergosterol. They reported that yeasts from family Cryptococcaceae produced no demonstrable sterol, with the exc tion of *Trigonopsis variabilis* A.T.C.C. 10,679.

**Determination of Ergosterol.** Procedures for assaying the ergost content of microbial preparations have been described by Bills et (1930); Pruess et al. (1931); Castille and Ruppol (1933); Bernhauer Patzelt (1935); Wenck et al. (1935*a, b*); Cavallito (1944); Stoudt Foster (1954); Kieber, Payne, and Appleton (1955); and others.

**Applications.** Ergosterol may be converted to vitamin $D_2$ by irra tion with ultraviolet rays. It may also be used as a raw material for synthesis of steroids through the use of chemical and microbiolog transformations.

### Some References concerning Vitamin Production by Yeasts and Yeast-like Organisms

Agarwala, S. C., and W. H. Peterson: *Arch. Biochem.,* 27: 327 (1950*a*).

——— and ———: *Arch. Biochem.,* 27: 316 (1950*b*).

Burkholder, P. R.: Synthesis of Riboflavin by a Yeast, *Proc. Nat. Acad. Sci.,* 166–172 (1943).

——: Influence of Some Environmental Factors upon the Production of Riboflavin by Yeast, *Arch. Biochem.,* **3**: 121–129 (1943).

——: Fermentation Process for the Production of Riboflavin (Vitamin B$_2$), U.S. Patent 2,363,227, Nov. 21, 1944.

ang, W., and W. H. Peterson: Factors Affecting the Biotin Content of Yeasts, *Jour. Bact.,* **58**: 33 (1949).

iao, J. S., and W. H. Peterson: *Jour. Agr. Food Chem.,* **1**(16): 1005 (1953).

le, J. K.: Process for Obtaining Crystalline Riboflavin, U.S. Patent 2,421,142, May 27, 1947.

den, E. L., Jr., D. N. Petsiavas, and J. Winoker: Microbiological Production of Riboflavin and Citric Acid from Citrus Molasses, *Jour. Agr. Food Chem.,* **2**(12): 632–638 (1954).

illiermond, A.: Sur un Champignon nouveau, parasite des capsules du Cotonnier, l'Eremothecium Ashbyii et ses relations possibles avec le Spermophthora Gossypii et les Ascomycetes, *Compt. rend.,* **200**: 1556–1558 (1935).

——, M. Fontaine, et A. Raffy: Sur l'existence dans l'Eremothecium Ashbyii d'un pigment jaune se rapportant au groupe des flavines, *Compt. rend.,* **201**: 1077–1080 (1935).

key, R. J.: Chapter in "Industrial Fermentations," Vol. 2, by L. A. Underkofler and R. J. Hickey, Chemical Publishing Co., New York, 1954.

nes, G. E., Jr.: Process for Recovery of Riboflavin, U.S. Patent 2,367,644, Jan. 16, 1945*a*.

——: Process for the Recovery of Riboflavin, U.S. Patent, 2,387,023, Oct. 16, 1945*b*.

lden, J. T., C. Furman, and E. E. Snell: *Jour. Biol. Chem.,* **178**: 789 (1949).

resztesy, J. C.: Production of Riboflavin from Fermentation Residues, U.S. Patent 2,355,220, Aug. 8, 1944.

wis, J. C.: Relationship of Iron Nutrition to the Synthesis of Vitamins by *Torulopsis utilis, Arch. Biochem.,* **4**: 217–228 (1944).

Millan, G. E.: Process for Recovering Crystalline Riboflavin, U.S. Patent 2,367,646, Jan. 16, 1945.

rimanoff, A., et A. Raffy: Obtention de la flavine à l'état cristallisé à partir d'Eremothecium Ashbyii, *Compt. rend.,* **206**: 1507–1509 (1938).

—— et ———: Obtention de Flavine a partir d'un ascomycete, *Eremothecium Ashbyii, Helv. Chim. Acta,* **21**: 1004–1006 (1938).

ra, K. K.: Part I.—Influence of Some Environmental Factors on the Growth & Riboflavin Production, *Jour. Sci. Ind. Research* (*India*), **11B**(3): 109–115 (1952).

vell, W.: *West Indian Bull.,* **16**: 152–159 (1917).

on, B. H., and M. J. Johnson: Factors Producing High Yeast Yields in Synthetic Media, *Jour. Bact.,* **57**: 235–246 (1949).

ifer, V. F., F. W. Tanner, Jr., C. Vojnovich, and D. H. Traufler: Riboflavin by Fermentation with *Ashbya gossypii, Ind. Eng. Chem.,* **42**: 1776–1779 (1950).

sma, H. D.: Production of Riboflavin, U.S. Patent 2,400,710, May 21, 1946.

lham, T. G.: Biological Production of Riboflavin, U.S. Patent 2,578,738, Dec. 18, 1951.

——: Microbial Synthesis of Riboflavin, *Econ. Botany,* **6**(2): 185–205 (1952). (Article contains 214 references.)

——, H. H. Hall, and V. F. Pfeifer: Production of Riboflavin (Vitamin B$_2$) with *Ashbya gossypii, U.S. Dept. Agr., Bur. Agr. and Ind. Chem., Mimeographed Circ. Ser.,* AIC 271, March, 1950.

—— and K. B. Raper: *Ashbya gossypii*—Its Significance in Nature and in the Laboratory, *Mycologia,* **42**(5): 603–623 (1950).

osa, M.: Synthesis of Riboflavin by Lactose-fermenting Yeasts, *Jour. Bact.,* **45**(5): 459–460 (1943).

lert, F. J.: Production of Riboflavin by Biochemical Methods, U.S. Patent 2,374,503, Apr. 24, 1945.

opfer, W. H.: La biotine, l'aneurine et le méso-inositol, facteurs de croissance pour *Eremothecium Ashbyii* Guilliermond. La biosynthès de la riboflavine, *Helv. Chim. Acta,* **27**: 1017–1032 (1944).

gh, K., P. N. Agarwal, and W. H. Peterson: *Arch. Biochem.,* **18**: 181 (1948).

Smiley, K. L., M. Sobolov, F. L. Austin, R. A. Rasmussen, M. B. Smith, J. M Lanen, L. Stone, and C. S. Boruff: Biosynthesis of Riboflavin, *L. bulg* factor, and Other Growth Factors, *Ind. Eng. Chem.*, **43**: 1380–1384 (1951)

Tanner, F. W., Jr., and J. M. Van Lanen: U.S. Patent 2,424,003, July 15, 1947.

———, C. Vojnovich, and J. M. Van Lanen: Riboflavin Production by *Ca* Species, *Science*, **101**(2616): 180–181 (February, 1945).

———, ———, and ———: Factors affecting riboflavin production by *Ashby* *sypii, Jour. Bact.*, **58**: 737–745 (1949).

Van Lanen, J. M.: *Arch. Biochem.*, **12**: 101 (1947).

———, H. P. Broquist, M. J. Johnson, I. L. Baldwin, and W. H. Peterson: Syn of Vitamin $B_1$ by Yeast, *Ind. Eng. Chem., Ind. Ed.*, **34**(10): 1244 (1942).

Wickerham, L. J., M. H. Flickinger, and R. M. Johnston: The Production of flavin by *Ashbya gossypii, Arch. Biochem.*, **9**(1): 95–98 (1946).

## Some References concerning Sterol Production by Microorganisms

Angeletti, A., and G. Tappi: Sulla biosintesi di steroli ad opera di alcuni cep penicilli, *Gazz. chim. ital.*, **77**: 112–114 (1947).

Appleton, G. S., R. J. Kieber, and W. J. Payne: The Sterol Content of Fung Screening of Representative Yeasts and Molds for Sterol Content, *Microbiol.*, **3**(4): 249–251 (1955).

Bennett, W. G.: Manufacture of Yeast, U.S. Patent 2,059,980, Nov. 3, 1936.

———: Manufacture of Yeast and Other Like Microorganisms, U.S. Patent 2,27 Mar. 17, 1942.

Bergmann, W.: The Plant Sterols, *Ann. Rev. Plant Physiol.*, **4**: 383–426 (1953).

Bernhauer, K., and G. Patzelt: Über Schimmelpilz-Sterine. I. Die Sterinbildun Aspergillus niger, *Biochem. Zeit.*, **280**: 388–393 (1935).

Bills, C. E., O. N. Massengale, and P. S. Prickett: Factors Determining the Ergos Content of Yeast. I. Species, *Jour. Biol. Chem.*, **87**: 259–264 (1930).

Birkinshaw, J. H., R. K. Callow, and C. F. Fischmann: CCXII. Studies in the chemistry of Microorganisms. XXII. The Isolation and Characterizatio Ergosterol from *Penicillium puberulum* Bainer Grown on a Synthetic Me with Glucose as Sole Source of Carbon, *Biochem. Jour.*, **25**: 1977–1980 (193

Castille and E. Ruppol: Ergosterol and Yeast, *Bull. acad. roy. méd. Belg.*, **13**: (1933).

Cavallito, C. J.: Ergosterol from Some Species of Penicillium, *Science*, **100**: (1944).

Dulaney, E. L., E. O. Stapley, and K. Simpf: Studies on Ergosterol Productio Yeasts, *Appl. Microbiol.*, **2**(6): 371–379 (1954).

Kieber, R. J., W. J. Payne, and G. S. Appleton: The Sterol Content of Fun Methods for Disrupting Cells, Extracting and Determining Sterols, *Appl. M biol.*, **3**(4): 247–248 (1955).

Maguigan, W. H., and E. Walker: Sterol Metabolism of Microorganisms. I. Ye *Biochem. Jour.*, **34**: 804–813 (1940).

Massengale, O. N., C. E. Bills, and P. S. Prickett: Factors Determining the Ergos Content of Yeast. II. Carbohydrate Sources, *Jour. Biol. Chem.*, **94**: 21 (1931).

Ottke, R. C., S. Simmonds, and E. L. Tatum: Deuteroacetate in the Biosynthes Ergosterol by Neurospora, *Jour. Biol. Chem.*, **186**: 581–589 (1950).

———, E. L. Tatum, I. Zabin, and K. Bloch: Isotopic Acetate and Isovalerate i Synthesis of Ergosterol by Neurospora, *Jour. Biol. Chem.*, **189**: 429–433 (195

Oxford, A. E., and H. Raistrick: CLVIII. Studies in the Biochemistry of M organisms. XXXIII. The Mycelial Constituents of *Penicillium brevi-compa* Dierckx and Related Species. Part I. Ergosteryl Palmitate, *Biochem. Jour.* 1176–1180 (1933).

Prickett, P. S., O. N. Massengale, W. M. Cox, Jr., and C. E. Bills: Factors D mining the Ergsterol Content of Fungi, *Proc. Soc. Exptl. Biol. Med.*, **27**: 702 (1930).

Pruess, L. M., E. C. Eichinger, and W. H. Peterson: The Chemistry of Mold Ti

III. Composition of Certain Molds with Special Reference to the Lipid Content, *Centr. Bakt. Parasitenk.*, Abt. II, **89**: 370 (1934).

—, H. J. Gorcica, H. C. Greene, and W. H. Peterson: Wachstum und Steringhalt gewisser Schimmelpitze, *Biochem. Zeit.*, **246**: 401–413 (1932).

—, W. H. Peterson, H. Steenbock, and E. B. Fred: Sterol Content and Antirachitic Activatibility of Mold Mycelia, *Jour. Biol. Chem.*, **90**: 369–384 (1931).

ıaswamy, S., and M. Sreenivasaya: Cultural Conditions Influencing the Ergosterol Content of Yeast, *Jour. Sci. Ind. Res.* (*India*): **5B**: 51 (1946).

ırd, K., and G. A. Grant: Ergosterol from the Mycelium of *Penicillium notatum* (Submerged Culture), *Science*, **104**: 459–460 (1946).

ıdt, T., and J. W. Foster: The Microbiological Synthesis of Ergosterol. I. Assay Procedure, *Appl. Microbiol.*, **2**: 385–387 (1954).

ret, C.: Sur un nouveau principe immediat de l'ergot de seigle l'ergosterine, *Compt. rend.*, **108**: 98 (1889).

ghelovici, M., and F. Serban: Contributions to the Study of the Biosynthesis of Sterols by *Aspergillus niger.*, *Acad. Rep. Populare Române, Bul. Stiint.*, **22**: 287–292 (1940).

— and ———: Contributions to the Study of the Biosynthesis of Sterols by *Aspergillus niger. II. Acad. Rep. Populare Române, Bul. Stiint.*, **23**: 436 (1941).

ıck, P. R., W. H. Peterson, and E. B. Fred: The Chemistry of Mold Tissue. IX. Cultural Factors Influencing Growth and Sterol Production of *Aspergillus fischeri*, *Centr. Bakt. Parasitenk.*, Abt. II, **92**: 330–338 (1935*a*).

—, ———, and H. C. Greene: The Chemistry of Mold Tissue. VIII. Innate Factors Influencing Growth and Sterol Production of *Aspergillus fischeri*, *Centr. Bakt. Parasitenk.*, Abt. II, **92**: 324–330 (1935*b*).

k, H. D., T. S. Oakwood, and F. C. Whitmore: Isolation of Ergosterol from *Penicillium notatum*, *Science*, **99**: 427–428 (1944).

koczy, G.: Process for Increasing the Ergosterol Content of Yeast, German Patent 720007, Class 6*a*, Group 20, Apr. 25, 1939.

## CHAPTER 11

# YEAST ENZYMES AND MINOR PRODUCTS

## YEAST ENZYMES

The different species of yeasts are sources of many different k enzymes, which function in their life activities. Representative e found in yeasts are listed in Table 72 (refer also to Chap. 5). polygalacturonase is one of the recently discovered enzymes. The e of the Krebs cycle are known to exist in *Saccharomyces cer* (DeMoss and Swim, 1957).

Certain yeast enzymes, such as lactase and invertase, are manufa industrially.

**Lactase.** This enzyme catalyzes the conversion of 1 molecule tose to 1 each of galactose and glucose.

At high concentrations, such as are obtained sometimes in ice and other dairy mixes, lactose tends to crytallize out. The consume think that he is eating sand or broken glass. In order to preve undesirable crystallization, lactase is added.

The best sources of lactase appear to be lactose-fermenting yeast as *S. fragilis, S. lactis, Candida spherica, C. pseudotropicalis,* and C (special strains).

A number of methods for producing lactase have been patented method patented by Myers (1956), the yeast (one of those men above) may be grown in a whey medium. The whey, derived cheddar- or cream-cheese manufacture, is treated to remove the coagulable protein by adjusting the pH to 4.5 and heating it at perature of 185 to 220°F. until coagulation is complete and by fil Ammonia in the amount of 0.1 per cent is added. The whey mediu prepared is cooled to 86°F. and inoculated with 10 per cent by we actively growing yeast. The temperature is kept at 86°F., and air plied to the medium at the rate of 0.1 volume per volume of mediu minute during a period of about 24 hr. The yeast cells propagated these conditions are separated from the spent medium by centri and washed with warm water. They are then quickly frozen at perature close to 0°F. to inactivate the zymase. The resultant la active, zymase-inactive product is dried in vacuum.

In another method, Myers and Stimpson (1956) propagate a la active yeast under growth-favoring conditions in a nutrient mediu sisting of deproteinated whey in which lactose is the chief carboh

TABLE 72. REPRESENTATIVE ENZYMES FROM YEAST *

| Enzyme | Substrate | Major products |
|---|---|---|
| rtase | Sucrose | Glucose and fructose |
| tase | Maltose | Glucose |
| ase | Lactose | Glucose and galactose |
| biase | Melibiose | Glucose and galactose |
| nuclease | Ribonucleic acid | Nucleotides |
| leotidase | Nucleotides | Nucleoside and phosphate |
| leosidase | Nucleosides | Purines or pyrimidines and ribose |
| sphatase | Phosphate esters (e.g., glycerophosphate) | Inorganic phosphate and alcohol |
| | Cocarboxylase | Thiamin phosphate and phosphate |
| phosphatase | Inorganic pyrophosphate | Phosphate |
| hol dehydrogenase | Ethanol | Acetaldehyde |
| ic dehydrogenase | Lactic acid | Pyruvic acid |
| ochrome c oxidase | Reduced cytochrome c | Oxidized cytochrome c |
| sephosphate hydrogenase | Diphosphoglyceraldehyde | Diphosphoglyceric acid |
| ow enzyme | Reduced coenzyme II | Coenzyme II |
| ochrome c reductase | Oxidized cytochrome c | Reduced cytochrome c |
| lase | Fructose diphosphate | Triose phosphates |
| boxylase | Pyruvic acid | Acetaldehyde and $CO_2$ |
| sphorylase | Glycogen or glucose phosphate | Glucose phosphate or glycogen |
| ose isomerase | Fructose phosphate | Glucose phosphate |
| sphoglucomutase | Glucose-1-phosphate | Glucose-6-phosphate |
| kinases | Fructose or glucose + ATP | Fructose or glucose phosphate |
| sphohexokinase | Fructose phosphate + ATP | Fructose or glucose phosphate |
| no acid deaminase-carboxylase | Amino acids | Alcohol, $NH_3$ and $CO_2$ |
| raginase | Asparagine | Aspartic acid |
| rtase | Aspartic acid | Fumaric acid |
| einase | Proteins | Peptides and amino acids |
| peptidase | Polypeptides | Lower peptides and amino acids |
| eptidase (several ecific enzymes) | Dipeptides (but not polypeptides) | Amino acids |
| ehyde mutase | Acetaldehyde | Alcohol and acetic acid |
| nsaminase | Glutamic + oxaloacetic acids | α-Ketoglutaric and aspartic acids |

Courtesy of Anheuser-Busch, Inc., Dried Yeasts and Derivatives Department, Louis, Mo.

source at a pH in the range of 3.5 to 7.5 and at a temperature of 100°F., harvest it, and dry it to obtain a lactase-active product.

Morgan (1955) patented a method for recovering a lactase-a zymase-inactive preparation. The lactase-active yeast is treated wi amount of ethanol equal to 1.5 to 3 times the weight of the yeast until a substantial proportion of the viable cells has been destroyed. product is then dried.

A method for producing *S. fragilis* with an optimum yield of lactas been patented by Young and Healey (1957).

**Invertase.** This enzyme causes the inversion of sucrose with the mation of glucose and fructose. It is used by confectioners, bakers sirup manufacturers.

The subject of invertase has been reviewed by Neuberg and Ro (1946).

**Yeast Polygalacturonase.** This enzyme, first reported by Luh Phaff in 1951 and extensively studied by Phaff and his associates, soluble extracellular enzyme elaborated by *S. fragilis* in complex as as synthetic, protein-free media. The enzyme appears to be constitu

The properties of yeast polygalacturonase (YPG) have been revie by Luh and Phaff (1954*a*, *b*). YPG appears to be a single peptic enz produced by the yeast in nearly pure form (Phaff and Demain, 1956 has a specific activity of 0.179 polygalacturonase units per mg. of pro (Demain and Phaff, 1954*a*). Although it produces di- and galactur acids from peptic acid, it is unable to attack digalacturonic acid and h differs from the fungal polygalacturonase. No pectinesterase activit associated with it.

A crude solution of the enzyme may be prepared (Luh and Pl 1954*a*) by growing *S. fragilis* strain 351 in the synthetic yeast nitro base (devised by Wickerham) containing 3 per cent glucose. The yea cultivated in 2.5-liter Fernbach flasks, holding 1 liter of medium e at 23 to 25°C. for 4 days. The cells are separated from the medium centrifuging. Toluene is added as a preservative to the solution cont ing the enzyme. Storage is carried out at 0°C. The enzyme may be centrated 13-fold by adsorbing it in the cold on pectic acid gel a pH of 3.0 and eluting it with 1 *N* acetate buffer at a pH of 5 (Dem and Phaff, 1954*a*).

The reactions concerned in the hydrolysis of pectic acid by yeast p galacturonase follow:

1. Rapid linear phase:

$$\text{Pectic acid} \rightarrow \text{tetra-} + \text{tri-} + \text{di-} + \text{galacturonic acids}$$

2. Slow linear phase:

$$\text{Tetragalacturonic acid} \rightarrow \text{tri-} + \text{galacturonic acids}$$

3. Very slow phase:

$$\text{Trigalacturonic acid} \rightarrow \text{di-} + \text{galacturonic acids}$$

optimum pH for the rapid linear phase is 4.4 (Demain and Phaff, *a*). Pentagalacturonic acid is formed during this phase, but it is olyzed into digalacturonic and trigalacturonic acids and tetragalac- nic and galacturonic acids combinations (Demain and Phaff, 1954*b*). slow linear phase starts after about 25 per cent of the pectic acid is olyzed. The optimum pH for this phase is 3.3 to 3.5. The third, or slow, phase commences after about 50 per cent of the pectic acid is olyzed and also proceeds best at the low pH. The reaction stops when er cent of the available bonds are broken.

e preparation of pure di- and trigalacturonic acids from pectic acid YPG has been described by Phaff and Luh (1952); and the prepara- of tetragalacturonic acid, by Demain and Phaff (1954*c*). The general of pectic enzymes, in which the authors propose to change the name st polygalacturonase" to "endopolygalacturonase," because the en- e attacks the chain randomly, has been reviewed by Demain and ff (1957).

## COENZYME SYSTEMS

tamins are important components of some coenzyme systems ble 73).

TABLE 73. VITAMINS AS COMPONENTS OF COENZYME SYSTEMS

| Vitamin | Coenzyme System of Which a Component |
|---|---|
| tinamide .... | Coenzyme I, or cozymase = diphosphopyridine nucleotide (DPN)<br>Coenzyme II = triphosphopyridine nucleotide (TPN) |
| othenic acid .. | Acetyl coenzyme A, an acetylation coenzyme |
| 'oxal ........ | Codecarboxylase |
| lavin ....... | Riboflavin coenzymes |
| nin ........ | Cocarboxylase = thiamin pyrophosphate (TPP) |

## YEAST INTERMEDIATE PRODUCTS

ast is an important source of certain organic chemicals. Table 74 nts some data relating to the preparation of yeast intermediates, e Fig. 37 shows some compounds which may be derived from yeast ylic acid.

ufer and associates (1953) have presented an interesting discussion is subject.

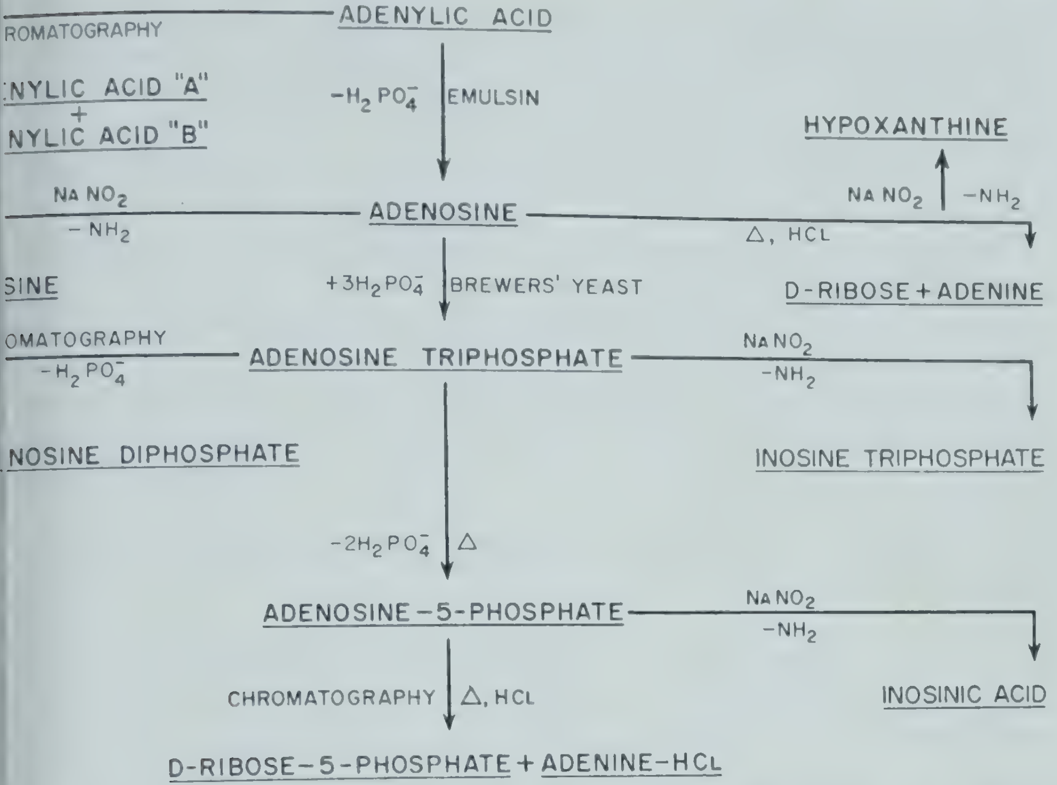
## ESTER FORMATION

eber (1922), Omelianski (1923), Yamada (1927), Gray (1949), achnick (1950), Peel (1950, 1951), Davies and associates (1951), a (1952), Tabachnick and Joslyn (1953*a*, *b*), Baba et al. (1953, 1954), tarelli (1955), and others have made valuable contributions concern- ster formation by microorganisms, principally yeasts.

sters, in particular ethyl acetate, are produced in largest amounts by

TABLE 74. SOME DATA RELATING TO THE PREPARATION OF SOME YEAST INTERM

| Intermediate | General method of preparation | Reference |
|---|---|---|
| Adenosine triphosphate ....... | Use of yeast enzymes | Laufer, L., D. R. Schwa E. D. Stewart: *Jour Food Chem.,* **1**: 91 (1 |
| Adenosine diphosphate ....... | ........... | Ostern, P., T. Baronows J. Terszakowec: *Zeit. p Chem.,* **25**: 258 (1938). |
| Adenosine monophosphate .... | ........... | Kornberg, A., and W. E. Jr.: *Jour. Biol. Chem* 481 (1951). |
| Coenzyme A ................... | Extraction | Beinert, H., et al.: *Jou Chem. Soc.,* **74**: 855 (1 |
| Diphosphopyridine nucleotide (coenzyme I) ............. | Extraction | Williamson, S., and D. E. *Jour. Biol. Chem.,* 13 (1940).<br>Laufer, L., D. R. Schwar E. D. Stewart: *Jour. Ag Food Chem.,* **1**: 91 (195 |
| Ergosterol .................. | Extraction | Dulaney, E. L., E. O. St and K. Simpf: *Appl. biol.,* **2**: 371 (1954).<br>Stoudt, T. H., and J. W. *Appl. Microbiol.,* **2**: 38 (1954).<br>Kieber, R. J., W. J. Payn G. S. Appleton: *Appl. biol.,* **3**: 247 (1955).<br>Bills, C. E., O. N. Masse and P. S. Prickett: *Biol. Chem.,* **87**: 259 (1 |
| Glutathione ................. | Extraction | Hopkins, F. G.: *Biochem.* **15**: 286 (1921); *Jour. Chem.,* **84**: 269 (1929).<br>Pirie, N. W.: *Biochem.* **24**: 51 (1930).<br>Laufer, L., D. R. Schwar E. D. Stewart: *Jour. Food Chem.,* **1**: 91 (195 |
| Nucleotides ................. | Extraction | Cohn, W. E.: *Jour. Am. Soc.,* **72**: 1471 (1949). |
| Ribonucleic acid ............ | Extraction | Laufer, L.: U.S. Patent 912, July 10, 1945. |


ADENYLIC ACID
ROMATOGRAPHY
:NYLIC ACID "A"
+
NYLIC ACID "B"
$-H_2PO_4^-$
EMULSIN
HYPOXANTHINE
NA NO$_2$
$-NH_2$
ADENOSINE
NA NO$_2$
$-NH_2$
△, HCL
SINE
$+3H_2PO_4^-$
BREWERS' YEAST
D-RIBOSE + ADENINE
OMATOGRAPHY
$-H_2PO_4^-$
ADENOSINE TRIPHOSPHATE
NA NO$_2$
$-NH_2$
NOSINE DIPHOSPHATE
INOSINE TRIPHOSPHATE
$-2H_2PO_4^-$
△
ADENOSINE-5-PHOSPHATE
NA NO$_2$
$-NH_2$
CHROMATOGRAPHY
△, HCL
INOSINIC ACID
D-RIBOSE-5-PHOSPHATE + ADENINE-HCL

37. Compounds derived from yeast adenylic acid. [*Courtesy of L. Laufer,*
*. Schwarz, and E. D. Stewart, Jour. Agr. Food Chem.,* 1: 91 (1953). *Copyright*
*by the American Chemical Society and reprinted by permission of the copy-*
*owner.*]

ies of the genus *Hansenula*, especially strains of *H. anomala.* Baba
52) reported on an anascosporogenous yeast, resembling *H. anomala*
*H. saturnus* in many respects, which produced large amounts of ethyl
ate. *Pichia* and other yeasts are known to produce esters.

nder normal conditions, the amount of ethyl acetate formed in a
ium reaches a maximum and then declines.

number of conditions affect the formation of esters by yeasts. These
ude nutrition, aeration, pH, temperature, and other factors. Ethyl
ate may be produced from a variety of carbon sources, such as glu-
, sucrose, molasses, and ethanol. Tabachnick and Joslyn (1953*a*)
ved that *H. anomala* produced ethanol from glucose, the alcohol being
verted in part under aerobic conditions to ethyl acetate. Baba and
ashi (1954) used blackstrap molasses as the carbon source and
ained highest yields in molasses media containing urea, ammonium
ate, ammonium acetate, or ammonium tartrate as nitrogen sources.
en their yeast, which was closely related to *H. anomala* except that it
ned no ascospores, was cultivated at 25 to 26°C. in a molasses medium
20° Balling) containing 0.5 per cent ammonium sulfate, the amounts
thyl acetate and ethanol produced on the sixth day after inoculation
1,450 mg. per 100 ml. (corrected) and 812 mg. per 100 ml.,
ectively.

uspensions of washed yeast cells have been used by a number of investigators to study the optimum conditions for ester formation mechanism of this reaction. Peel (1951) observed that cell sus of *H. anomala* formed esters under aerobic conditions from a containing ethanol and acetic acid at an optimum pH of 4.5 to 5 a ethanol alone at a pH presumably in the range of 2.5 to 3. Tab and Joslyn (1953*b*), using cell suspensions of *H. anomala,* found pH of 2.1 to 2.6 and a temperature of about 19°C. were optimum accumulation from ethanol.

Cantarelli (1955), using washed cell suspensions of *Hansenul* observed that esterification capacity was increased by growing th in a medium enriched with pantothenate; that potassium and ma ions favored synthesis whereas calcium ions reduced it; that oxy necessary for ester formation and that cyanide inhibited it; a arsenate, azide, and 2-,4-dinitrophenol acted as poisons towar phorylation in the respiratory cycle and, accordingly, inhibite synthesis.

The exact mechanism for the formation of ethyl acetate from is not known. Tabachnick and Joslyn (1953*b*) reported that ester not concerned in the synthesis of ethyl acetate, for the enzyme wa ited at pH 2.1 and the ester accumulated at pH 6.7 in the pres diisopropyl fluorophosphate (an esterase inhibitor). They were opinion that ester formation by *H. anomala* was an energy- reaction. Cantarelli (1955) suggested that esters may be for accordance with the following reactions:

$$C_2H_5OH + DPN^+ \rightleftharpoons CH_3CHO + DPN + H^+$$

$$CH_3CHO + CoA\cdot SH + DPN^+ \rightleftharpoons CH_3CO\cdot SCoA + DPNH + H^+$$

$$(\text{or } CH_3CHO + HPO_4^{--} \rightleftharpoons CH_3COOPO_3^{--};$$

$$CH_3COOPO_3^{--} + CoA\cdot SH \rightleftharpoons CH_3CO\cdot SCoA + HPO_4^{--})$$

$$CH_3CO\cdot SCoA + C_2H_5OH \rightleftharpoons CH_3COOC_2H_5 + CoA\cdot SH$$

Peel (1951) and Tabachnick and Joslyn (1953*b*) concluded th acetate portion of the ester was formed within the cell and not fro acetic acid in the medium. The latter authors found that acetic ac toxic in concentrations ranging from 0.01 *M* to 0.05 *M* at pH 2.1.

A chromatographic technique for the identification of ethyl aceta other esters was described by Cantarelli (1955, 1956).

## PRODUCTION OF POLYHYDRIC ALCOHOLS BY OSMOPHILIC YEASTS

Spencer and Sallans (1956) reported on the production of gl erythritol, D-arabitol, and mannitol, along with ethanol, by osm yeasts isolated from fermenting honey, brood-comb pollen, drie flower parts, and other sources. The basal medium used by the tained 20 per cent glucose. The effects of added yeast extract and ent nitrogen sources were determined during the studies. The test carried out in 250- or 500-ml. Erlenmeyer flasks incubated at 30 r Brunswick or Gump shakers. The analytical methods used have n described by Neish (1952).

total of 79 strains of osmophilic yeasts were studied for polyhydric hol production. Most (24 out of 27) of the fast-growing cultures lays or less) were included in the 39 strains which produced glycerol D-arabitol. In the remaining 40 strains, there were 13 which produced erol and erythritol; 10 which produced glycerol; 6 which produced erol, D-arabitol, and erythritol; 6 which produced D-arabitol; 3 which luced erythritol; and 2 which produced erythritol and D-arabitol. nnitol was produced in small amounts by some of the strains.

n media containing 20 per cent glucose, 0.4 per cent yeast extract and plementary sources of nitrogen, the results shown in Table 75 were

TABLE 75. EFFECT OF AMMONIUM PHOSPHATE, AMMONIUM SULFATE, AND UREA ON GLYCEROL AND D-ARABITOL PRODUCTION *, †

| upplementary itrogen source | Average rate ‡ of glucose utilization, mg./ml./day | Final glycerol,‡ mg./ml. | Final D-arabitol,‡ mg./ml. | Ratio of glycerol to D-arabitol |
|---|---|---|---|---|
| e .............. | 7.8 | 6 | 94 | 0.064 |
| a 0.114% ........ | 13.8 | 22 | 76 | 0.29 |
| $I_4)_2SO_4$ 0.05% ... | 10.8 | 14 | 56 | 0.25 |
| $H_2PO_4$ 0.09% ... | 11.5 | 7 | 66 | 0.11 |

J. F. T. Spencer and H. R. Sallans, *Can. Jour. Microbiol.*, 2: 72 (1956).
150-ml. medium containing 20 per cent glucose and 0.4 per cent yeast extract in nl. Erlenmeyer flasks, on New Brunswick shaker at 160 r.p.m. Temperature .

Average of two flasks.

ined. It will be observed that the highest yield of D-arabitol and the est yield of glycerol were obtained when no supplementary source of ogen was added and that the use of urea favored the highest yield of erol.

was found that increasing the amount of yeast extract to 1 per cent lted in an increased yield of glycerol, ratio of glycerol to D-arabitol, rate of glucose utilization. Similar results were secured when small ounts of urea, ammonium sulfate, ammonium tartrate, or ammonium sphate were added to the medium. The effect was greatest with urea. ncreased aeration of the medium resulted in increased yields of erol, reduced yields of ethanol, and no significant change in the yield -arabitol.

pencer and Sallans concluded that osmophilic yeasts fermented con- rated glucose solutions rapidly with about 60 per cent of the sugar ig converted to glycerol, D-arabitol, erythritol, and mannitol and that comparative yields of these alcohols may be altered greatly by strain ction and the exact control of the conditions of growth.

Roxburgh and associates (1956) reported on the recovery of pol alcohols from yeast fermentations.

Oxygen tension and inorganic phosphate concentration exert r effects on the production of glycerol, arabitol, and ethanol by *Sa* *myces rouxii* strain P$_3$a, an osmophilic yeast, according to Spen Shu (1957).

Increasing the oxygen tension resulted in an increase in the yi glycerol and arabitol and a decrease in the yield of ethanol.

Increasing the orthophosphate concentration (Table 76) resu

Table 76. Effect of Disodium Phosphate on Polyhydric Alcohol Produ
(Basal medium 20 per cent glucose + 1.0 per cent yeast extract. Incubated rotary shaker of 1-in. eccentricity, speed 230 r.p.m., temperature 30°C.

| $Na_2HPO_4$, moles/liter (× 1,000) | Maximum rate of glucose utilization, mg./ml./day | Polyhydric alcohols, mg./ml. | | Ethanol, mg | |
|---|---|---|---|---|---|
| | | 2 days | 4 days | 2 days | 4 d |
| None | 67 | 30 | 82 | 17 | |
| 0.52 | 74 | 28 | 67 | 22 | |
| 1.04 | 86 | 26 | 50 | 26 | 1 |
| 2.08 | 99 | 25 | 31 | 35 | 2 |

* J. F. T. Spencer and P. Shu, *Can. Jour. Microbiol.*, **3**: 559 (1957).

decreased yields of the polyhydric alcohols but increased the yie ethanol, the rate of glucose utilization, and the respiratory quotient effect was most pronounced at p$O_2$ of 280 mm. of mercury (a critica gen tension). The changes in the yields of alcohols with changes phosphate concentration were much less when the p$O_2$'s were grea less than this. At lower oxygen tensions, the yields of polyhydric al were low, and those of ethanol were high at all phosphate concentra whereas at very high oxygen tensions, the effects were just the op

The authors believe that the effects may be explained on the ba the oxidative phosphate–lack theory of the Pasteur effect discuss Johnson (1941) and Lynen (1941) and supported by Holzer and I (1953). According to this theory, the concentration of inorganic phate is a limiting factor in respect to the activity of glyceraldeh phosphate dehydrogenase. An increase in the activity of this e results in the conversion of additional amounts of triosephosph ethanol, with a reduced yield of glycerol. With an increase in the o tension, more orthophosphate is esterified per mole of glucose dissim and the amount of inorganic phosphate is reduced, inhibitin glyceraldehyde-3-phosphate dehydrogenase and reducing the rate c cose dissimilation.

### Some References concerning Yeast Products

DeMoss, J. A., and H. E. Swim: Quantitative Aspects of the Tricarboxylic Aci in Bakers' Yeast, *Jour. Bact.*, **74**: 445–451 (1957).

, C. G.: The Industrial Utilization of Yeasts, *Economic Botany,* **12**(2): 145–
63 (1958).

r, L., D. R. Schwarz, and E. D. Stewart: Yeast a Source of Biochemical Inter-
ediates, *Jour. Agr. Food Chem.*, **1**: 91–95 (April, 1953).

erg, C., and I. S. Roberts: Invertase, *Scientific Report Series,* No. 4, Sugar
esearch Foundation Inc., New York, September, 1946.

## Some References concerning Lactase

an, E. R.: Lactase Enzyme Preparation, U.S. Patent 2,715,601, Aug. 16, 1955.

s, R. P.: Drying of Yeast to Inactivate Zymase and Preserve Lactase, U.S.
atent 2,762,748, Sept. 11, 1956.

– and E. G. Stimpson: Production of Lactase, U.S. Patent 2,762,749, Sept. 11,
956.

g, H., and R. P. Healey: Production of *Saccharomyces fragilis* with an Opti-
num Yield of Lactase, U.S. Patent 2,776,928, Jan. 8, 1957.

## Some References concerning Yeast Polygalacturonase

in, A. L., and H. J. Phaff: Composition and Action of Yeast Polygalacturonase,
*ature,* **174**: 515 (1954*a*).

– and ———: Hydrolysis of the Oligogalacturonides and Pectic Acid by Yeast
olygalacturonase, *Jour. Biol. Chem.,* **210**: 381–393 (1954*b*).

– and ———: The Preparation of Tetragalacturonic Acid, *Arch. Biochem. and
iophys.,* **51**: 114–121 (1954*c*).

– and ———: Recent Advances in the Enzymatic Hydrolysis of Pectic Sub-
tances, *Wallerstein Labs. Communs.,* **20**(69): 119–140 (1957).

B. S., and H. J. Phaff: Properties of Yeast Polygalacturonase, *Arch. Biochem.
nd Biophys.,* **48**: 23–37 (1954*a*).

– and ———: End Products and Mechanism of Hydrolysis of Pectin and Pectic
cid by Yeast Polygalacturonase (YPG), *Arch. Biochem. and Biophys.,* **51**: 102–
13 (1954*b*).

H. J., and A. L. Demain: The Unienzymatic Nature of Yeast Polygalacturo-
ase, *Jour. Biol. Chem.,* **218**: 875–884 (1956).

– and B. S. Luh: The Preparation of Pure Di- and Trigalacturonic Acids,
*rch. Biochem. and Biophys.,* **36**: 231, 232 (1952).

## ome References concerning Ethyl Acetate and Its Formation by Yeasts

T.: Studies on Production of Ester by Yeasts. I. On a New Species of Ethyl
cetate-forming Yeasts, *Bull. Fac. Eng., Hiroshima Univ.,* No. 1, April, 1952,
p. 111–115.

– and M. Hayashi: Fundamental Studies on the Manufacture of Ethyl Acetate
om Blackstrap Molasses by a Fermentation Process. I. Influence of Nitrogenous
latters on the Ethyl Acetate Fermentation of Molasses with a New Species of
east, *Bull. Fac. Eng., Hiroshima Univ.,* **3**(2): 243–252 (September, 1954).

–, ———, and I. Hori: Studies on the Production of Esters by Yeasts. II. On the
ormation of Ethyl Acetate and the Oxidation of Alcohols, *Bull. Fac. Eng.,
iroshima Univ.,* No. 2, February, 1953, pp. 33–40.

arelli, C.: La formazione degli esteri volatili nel corso della fermentazione
lcolica, *Ann. microbiol.,* **6** (fasc. 6): 219–241 (1955). (Contains rather exten-
ive bibliography.)

–: L'identificazione cromatografica degli esteri nel vino e nei fermentati alcolica,
*stratto dagli Ann. fac. agrar. univ. studi Perugia,* **10**: 1–21 (1955).

–: A Simple Chromatographic Technique for the Identification of Ethyl Ace-
ate and Other Esters in a Cultured Medium, *Antonie van Leeuwenhoek Jour.
icrobiol. Serol.,* **22**: 47, 48 (1956).

es, R., E. A. Falkiner, J. F. Wilkinson, and J. L. Peel: Ester Formation by

Yeasts. 1. Ethyl Acetate Formation by *Hansenula* Species, *Biochem. Jo* 58–61 (1951).

Gray, W. D.: Initial Studies on the Metabolism of *Hansenula anomala* ( Sydow, *Am. Jour. Botany,* **36**: 475–480 (1949).

Omelianski, V. L.: Aroma Producing Microorganisms, *Jour. Bact.,* **8**: 393–419

Peel, J. L.: Ester Formation by Yeasts. 2. Formation of Ethyl Acetate by Suspensions of *Hansenula anomala, Biochem. Jour.,* **49**: 62–67 (1951).

Tabachnick, J.: The Chemistry and Physiology of Ester Production by *Ha anomala,* Ph.D. thesis, University of California, Berkeley, Calif., 1950.

——— and M. A. Joslyn: Formation of Esters by Yeast. I. The Production o Acetate by Standing Surface Cultures of *Hansenula anomala, Jour. Ba* 1–9 (1953).

——— and ———: Formation of Esters by Yeast. II. Investigations with Suspensions of *Hansenula anomala, Plant Physiol.,* **28**: 681–692 (1953).

Weber, U.: Beitrag zur Kenntnis der Esterbilden den Hefen, *Biochem. Zei* 208–216 (1922).

Yamada, M.: On the Mechanism of Fruit-ester Formation by *Willia anoma Jour. Agr. Chem. Soc. Japan,* **3**(4–6): 73–76 (1927).

## Some References concerning Polyhydric Alcohols

Holzer, H., und E. Holzer: Bestimmung stationärer Triosephosphat Konzentra in lebender Hefe. Ein Beitrag zum Mechanismus des Pasteur-Effektes, *Seyler's Zeit. physiol. Chem.,* **292**: 232–239 (1953).

Johnson, M. J.: The Role of Aerobic Phosphorylation in the Pasteur Effect, *S* **94**: 200–202 (1941).

Lynen, F.: Über den aeroben Phosphatbedarf der Hefe, Ein Beitrag zur Ke der Pasteurischen Reaktion, *Ann.,* **546**: 120–141 (1941).

Neish, A. C.: "Analytical Methods for Bacterial Fermentations," 2d rev. ed., Na Research Council of Canada, Prairie Regional Laboratory, Saskatoon, C 1952.

Roxburgh, J. M., J. F. T. Spencer, and H. R. Sallans: Recovery of Polyhydric hols from Yeast Fermentations, *Can. Jour. Technol.,* **34**: 248–253 (1956).

Spencer, J. F. T., J. M. Roxburgh, and H. R. Sallans: Factors Influencing th duction of Polyhydric Alcohols by Osmophilic Yeasts, *Jour. Agr. Food* **5**(1): 64 (1957).

——— and H. R. Sallans: Production of Polyhydric Alcohols by Osmophilic *Can. Jour. Microbiol.,* **2**: 72–79 (1956).

——— and P. Shu: Polyhydric Alcohol Production by Osmophilic Yeasts: Eff Oxygen Tension and Inorganic Phosphate Concentration, *Can. Jour. Mici* **3**: 559–567 (1957).

## CHAPTER 12

# THE BACTERIA

cteria, like molds, because of their great range of types and the
ty of their action, may be very useful, extremely harmful, or, so
s present knowledge goes, of no particular significance to man. It is
known that bacteria cause diseases in animals and in plants and
mposition of foodstuffs and are responsible for many biochemical
ges in nature. Some of the useful applications of bacteria are con-
ed in the chapters that follow.

e bacteria that are used in industry may be divided by their relation
ee oxygen into two main groups: the anaerobic bacteria and the
ic bacteria. The former group, which may be considered to include
the microaerophiles, includes the largest number of useful types of
ria from the industrial standpoint. Bacteria active in producing
nol, acetone, ethanol, isopropanol, lactic acid, fermented milk prod-
cheese, and other substances of recognized or potential value are
ded in this group, although not all the organisms concerned in the
ction of some of these compounds are anaerobic in character. The
nding aerobic bacteria include the well-known *Acetobacter* group
cteria, some of which have the ability to produce acetic acid, glu-
acid, dihydroxyacetone, sorbose, and other substances from suitable
rates by oxidative means. Among the aerobes, also, may be placed
acteria concerned with vitamin $B_{12}$ production and other useful
ntations.

le 77 gives data concerning some important fermentation bacteria,
rmentations with which they are associated, their oxygen relation-
and the optimum temperatures for their growth and biochemical
ty.

le 78 lists some of the products of bacterial fermentation, some of
aterials from which these are formed, and the general class of the
ria concerned.

TABLE 77. IMPORTANT FERMENTATION BACTERIA: FERMENTATIONS, RELA
TO OXYGEN, AND OPTIMUM TEMPERATURES FOR GROWTH

| Family | Genus and species | Fermentations with which commonly associated | Relationship to air * |
|---|---|---|---|
| Pseudomonadaceae ....... | *Acetobacter roseus* | Gluconic acid | Aerobic |
| | *A. suboxydans* | Dihydroxyacetone, sorbose, acetic acid, gluconic acid, etc. | Aerobic |
| | *A. xylinum* | Sorbose, acetic acid, etc. | Aerobic |
| | *Bacterium curvum* | Vinegar (quick process) | Aerobic |
| | *Bact. orleanense* | Vinegar (Orleans and quick processes) | Aerobic |
| | *Bact. schutzenbachii* | Vinegar (quick process) | Aerobic |
| Lactobacillaceae Orla-Jensen: | | | |
| Tribe Streptococceae ... | *Leuconostoc mesenteroides* | Sauerkraut | Facultative aerobic |
| | | Dextrans | |
| | *Streptococcus lactis* | Lactic acid | Facultative aerobic |
| Tribe Lactobacilleae .... | *Lactobacillus acidophilus* | Acidophilus milk | Microaerophilic |
| | *L. bulgaricus* | Milk products, lactic acid | Aerobic to anaerobic |
| | *L. brevis* | Milk products, sauerkraut, ensilage, etc. | |
| | *L. casei* | Lactic acid | Microaerophilic |
| | *L. delbrueckii* | Lactic acid | Microaerophilic |
| | *L. leichmannii* | Lactic acid | Microaerophilic |
| | *L. plantarum* | Sauerkraut, pickles, milk products, etc. | Microaerophilic |
| Propionibacteriaceae ..... | *Propionibacterium freudenreichii* | Swiss cheese, propionic acid | Anaerobic |
| | *P. shermanii* etc. | Propionic acid, etc. | Anaerobic |
| Bacillaceae Fischer: | | | |
| Genus I ............... | *Bacillus macerans* Schardinger | Ethanol-acetone | Aerobic |
| | *B. acetoethylicus* Northrop et al. (probably identical with *B. macerans*) | Ethanol-acetone | Aerobic |
| Genus II ............... | *Clostridium acetobutylicum* McCoy, Fred, Peterson, and Hastings | Butanol-acetone | Anaerobic |
| | *Cl. butyricum* Prazmowski | Butanol-acetone | Anaerobic |
| | *Cl. felsineum* | Retting, butanol-acetone | Anaerobic |

---

* Data secured from "Bergey's Manual of Determinative Bacteriology," 7th ed., The Wi
Wilkins Company, Baltimore, 1957, and elsewhere.

TABLE 78. PRODUCTS OF BACTERIAL FERMENTATION

| rmentation product | Materials from which formed | Organisms causing change |
|---|---|---|
| ldehyde | Sugars, ethanol, pyruvic acid, etc. | Acetic acid and butanol-acetone bacteria, *Escherichia coli*, etc. |
| acid | Ethanol, glucose, cellulose, etc. | Acetic acid, heterofermentative lactic acid, butanol-acetone, isopropanol, propionic acid, and cellulose-fermenting bacteria, *E. coli* |
| acetic acid | Acetic acid | Butanol-acetone bacteria |
| ne | Starch, sugars, acetoacetic acid, acetic acid, isopropanol | Butanol-acetone, ethanol-acetone, isopropanol, and acetic acid bacteria |
| lmethylcarbinol | 2:3-Butyleneglycol, sugars, pyruvic acid, etc. | *Aerobacter aerogenes*, butanol-acetone and acetic acid bacteria |
| ol | Starch, sugars, butyric acid, etc. | Butanol-acetone and isopropanol bacteria |
| ic acid | Starch, sugars, cellulose, acetic acid, etc. | Butyric acid and cellulose-fermenting bacteria |
| tyleneglycol | Glucose, diacetyl | *A. aerogenes* |
| n dioxide | Starch, sugars, etc. | Butanol-acetone, *A. aerogenes*, *E. coli*, and many other bacteria |
| roxyacetone | Glycerol | Acetic acid bacteria |
| ol | Sugars, starch, cellulose, acetaldehyde, etc. | *Termobacterium mobile* Lindner, butanol-acetone, ethanol-acetone, heterofermentative lactic acid and cellulose-fermenting bacteria; *E. coli*, etc. |
| c acid | Glucose, cellulose, etc. | Butyric, cellulose-fermenting bacteria, *E. coli*, etc. |
| se | Mannitol | Acetic acid bacteria |
| onic acid | Galactose | Acetic acid bacteria |
| ose | Dulcitol | Acetic acid bacteria |
| ic acid | Glucose | Acetic acid bacteria |
| ol | Glucose, etc. | Heterofermentative lactic acid bacteria |
| ophosphoric acid | Triosephosphoric acid | Lactic acid bacteria, *E. coli* |
| diphosphate | Glucose | Lactic acid bacteria, *E. coli* |
| en gas | Glucose, lactose, etc. | Butyric acid bacteria, colon-aerogenes group, and other bacteria |
| anol | Starch, sugars | Isopropanol-acetone bacteria |
| gluconic acid | Gluconic acid (glucose) | Acetic acid bacteria |
| gluconic acid | Gluconic acid (glucose) | Acetic acid bacteria |
| cid | Glucose, fructose, etc. | Acetic acid bacteria |
| acid | Starch, sucrose, glucose, fructose, etc. | Lactic acid, propionic acid, and cellulose-fermenting bacteria; *E. coli*, etc. |
| l | Fructose | Heterofermentative lactic acid bacteria |
| ic acid | Mannose | Acetic acid bacteria |
| e | Mannitol | Acetic acid bacteria |
| glyoxal | Sugars, hexosediphosphate, etc. | Acetic acid, butanol-acetone, lactic acid and propionic acid bacteria; *E. coli* |
| oglyceric acid | Sugars, hexosediphosphate | Lactic acid bacteria, *E. coli* |
| ol | Propionic acid | Butanol-acetone bacteria |
| ic acid | Glucose, propanol, lactic acid, glycerol, pyruvic acid | Propionic acid and acetic acid bacteria |
| aldehyde | Glucose, glycerol | Propionic acid bacteria |
| ne glycol | Rhamnose | *Bacterium rhamnosifermentans* |
| acid | Sugars | Butyric, lactic, and propionic acid bacteria; *E. coli* |
|  | Sorbitol | Acetic acid bacteria |
| acid | Glucose, fructose, lactose, and other sugars | Propionic acid bacteria, *E. coli*, etc. |
| acid | Glucose | *A. suboxydans* |

## CHAPTER 13

# THE ACETONE-BUTANOL FERMENTATION

There are several closely related fermentations in which aceto ethanol occur as end products. One of these is an industrial fermen in which the chief neutral solvents of importance are butanol and ac another of these is also an industrial fermentation in which the pri neutral solvents are butanol and isopropanol; and still another is mentation of no present industrial importance in which aceton ethanol are the main end products.

**Historical.** Normal butyl alcohol was discovered as a reg occurring constituent of fusel oil by Wurtz in 1852. Pasteur, ho was the first investigator to show that butyl alcohol was a direct p of fermentation. His findings were based on the results of the b fermentation of lactic acid and calcium lactate. In a report annou his discovery he said, “M. Pasteur . . . croit pouvoir affirmer que l’ butylique est un produit ordinaire de la fermentation butyrique.”

During the period between 1876 and 1910, researches concerne butanol were carried out by Fitz (1882, 1884), Gruber (1887), Gri (1893), Beijerinck (1893), Duclaux (1895), Emmerling (1897), berger and Schattenfroh (1902), Winogradsky (1902), Schar (1905), and others.

The need for synthetic rubber supplied the impetus which resul the first successful commercial process (Arzberger et al., 1920; G 1928; Kelly, 1936). Significant research was carried out by the fi Strange and Graham, Ltd., in England. which employed Perkin, mann, Fernbach, Schoen, and others. Weizmann left the employ o firm in 1912 and not long afterward isolated a starch-fermenting ana which was capable of producing excellent yields of acetone and bu This isolate later became known as *Clostridium acetobutylicum* mann and was used in industrial processes.

The first successful commercial process was established in Engla the firm of Strange and Graham, Ltd., during the years 1913 and Soon thereafter plants were placed in operation in Canada, the States, and India. It was the acetone which was of primary conc this time, because of its use in the making of explosives and for purposes. With the termination of World War I, the plants were because acetone was no longer needed in large quantities. It was no however, before a demand arose for *n*-butanol in the manufactu automobile lacquers.

uring the year 1919 several papers concerning the acetone and ace--butanol fermentations were published. Nathan described the manuure of acetone by the Weizmann process from such raw materials as and horse chestnuts. Gill described in detail the acetone fermentaby the Weizmann organism and its technical application. In another rt he discussed the production of acetone and $n$-butyl alcohol from e chestnuts by fermentation. Features of the fermentation of horse tnuts, such as the long period of fermentation, frothing during fertation and distillation, and the unhealthy appearance of the bacteria ascribed to the presence of aesculic acid or aesculin. By removing uch of the husk as possible, the fermentation was improved. Yields horse chestnuts averaged about 18 per cent on the basis of dry meal mparison to 24 per cent from maize.

## INDUSTRIAL PRODUCTION FROM STARCHES

modern industrial process for manufacturing acetone and butanol starches was reported by Beesch (1953). The description which ws is based on his report.

**oculum Preparation.** The spores of *Clostridium acetobutylicum*, h are used for starting the culture, are maintained on a mixture of l soil, sand, and carbonate. Some of them are added to a sterilized or potato mash, containing about 4 per cent starch, in a test tube. latter with its contents is placed in a bath of boiling water for 90 sec. , depending on the size of the tube and the nature of the mash. The erature of the tube is reduced to 98°F., after which it is incubated for 24 hr. at 98°F. The contents of the tube are used to inoculate l. of sterilized 8 per cent corn mash in a 1,000-ml. Erlenmeyer flask. in turn is incubated for 24 hr. at 98°F. and provides inoculum (3 per for 3,000 ml. of sterilized mash of the same composition.

nples from the first and second stages of the laboratory inocula are l out to determine whether facultative anaerobes are present as minants. Should they be found, then new starters must be pre-.

contents of the last culture vessel are used to seed about 4,000 gal. rilized 8 per cent corn mash contained in a 5,000-gal. jacketed iron which is provided with an inoculation device and an agitator. The re is mixed with the mash. A blanket of sterilized inert gas (which be fermentation gas) is maintained over the seeded mash in order rnish anaerobic conditions and to reduce the chances of contaminarom the environment.

progress of fermentation in the 5,000-gal. tank is followed care-Observations are made for gas formation, pH, titrable acidity, and rature at regular intervals. Likewise, samples are plated out from nd the previous laboratory inoculum to make sure that microaero-and anaerobes are absent.

mentors, which are cylindrical, spherical, or spheroid in shape, are lated with 0.5 to 3 per cent of inoculum from the 5,000-gal. tank, but only after the titrable acidity drops and there is satisfactory evi that the inoculum is free from infection and normal.

**Preparation of the Fermentation Mash.** When corn is used a raw material, it is screened and passed over a magnet to remove dus iron. If economically feasible, the germ may be removed as a sour oil. The corn is ground next to a fine condition in roller or hammer The ground material is mixed with water to yield an 8 to 10 per cent centration of corn. The mash is cooked in a continuous sterilizer, as that described by Carnarius (1947) or by Pfeifer and Vojn (1952).

In the cooking process, the whole cooking system is filled with the which is raised to a temperature of 250 to 260°F. and held at this perature with recirculation for about 1 hr. The process then bec continuous by the addition of fresh slurry and the withdrawal o sterilized product. The mash may or may not be diluted by the add of sterile water at 180°F., and it is then cooled by passage throu series of double-pipe coolers to a fermentation temperature of 98°F. cooled mash is collected in the fermentors, which are of 60,00 500,000-gal. capacity.

**Filling the Fermentors.** The culture is usually added to the mentor at the start. In one method of filling, known as the "def filling method," the fermentor is then partly filled with sterilized which permits a higher concentration of cells to be built up within a period of time, which in turn accelerates the final fermentation and to reduce the chances of contamination build-up. In the other me the tank is entirely filled with mash after the addition of the starter

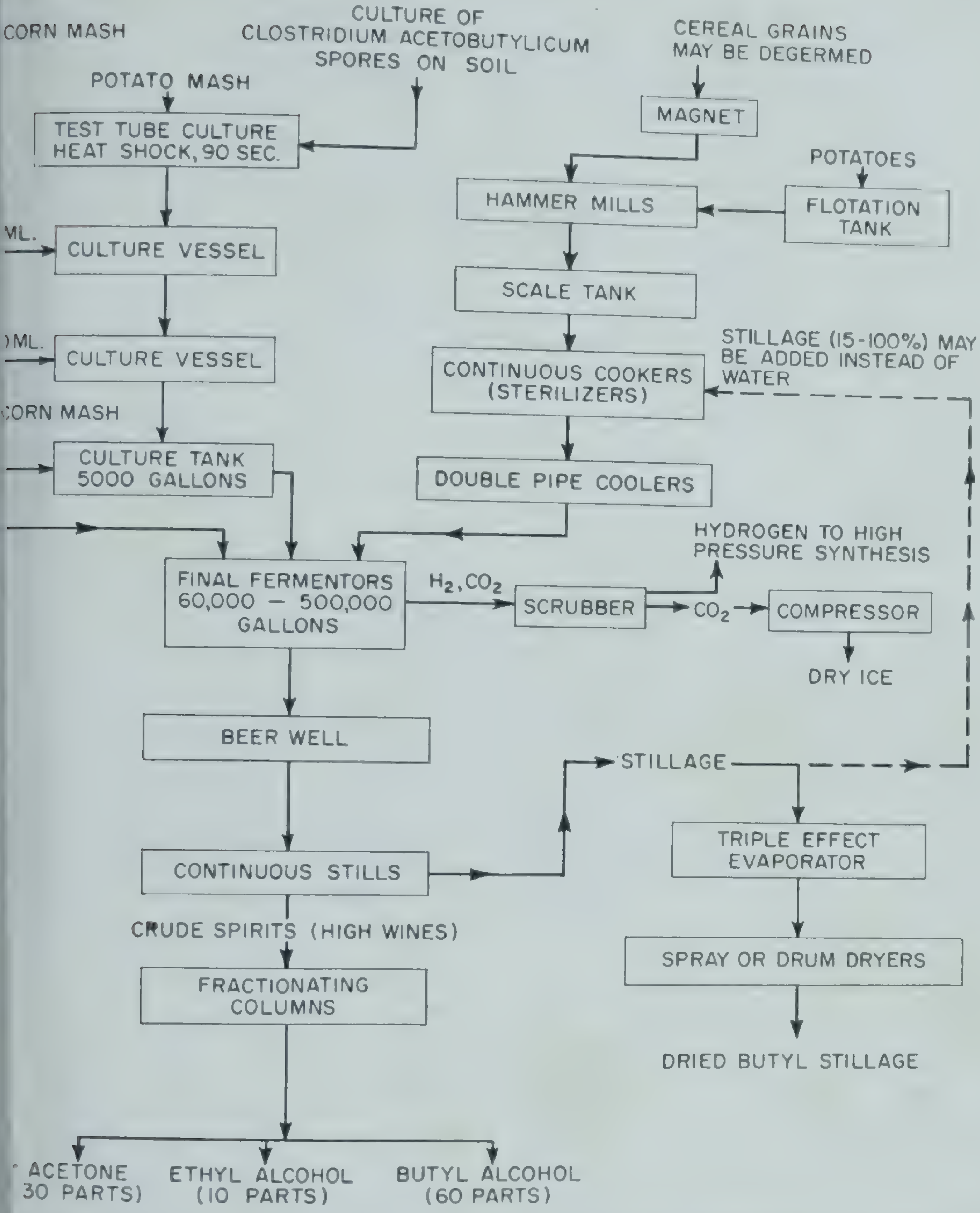
**Fermentation.** A number of conditions are optimum for the fer tation. The temperature is best at 98°F., but the fermentation ap to be normal in the range of 93 to 106°F. There may be a loss of ac at the higher temperatures and a change in solvent ratio. The pH a beginning of fermentation is usually 6 to 6.5 and at the end 4.2 to The time of fermentation is generally 50 to 60 hr.

**Flow Diagram.** A flow diagram of a modern typical acetone-but fermentation is shown in Fig. 38.

**Use of Stillage.** Beesch (1953) reported that either ethyl grain age or butyl grain stillage may be used as a replacement for part o of the water used in diluting the mash. Advantages cited are a b than normal fermentation, a saving in water and steam, and the redu of foaming in the fermentors. Patents concerning the use of stillage been issued to Hancock (1934), McCutchan and Arzberger (1935), Legg and Stiles (1937).

## INDUSTRIAL PRODUCTION FROM MOLASSES

**Organisms Producing.** The bacteria used for the commercial pro tion of acetone and butanol from saccharine raw materials are almos species of the genus *Clostridium*, as is indicated in Table 79. Solven different ratios are produced by these bacteria.


CORN MASH
CULTURE OF CLOSTRIDIUM ACETOBUTYLICUM SPORES ON SOIL
CEREAL GRAINS MAY BE DEGERMED
POTATO MASH
TEST TUBE CULTURE HEAT SHOCK, 90 SEC.
MAGNET
POTATOES
HAMMER MILLS
FLOTATION TANK
ML.
CULTURE VESSEL
SCALE TANK
)ML.
CULTURE VESSEL
STILLAGE (15-100%) MAY BE ADDED INSTEAD OF WATER
CONTINUOUS COOKERS (STERILIZERS)
CORN MASH
CULTURE TANK 5000 GALLONS
DOUBLE PIPE COOLERS
HYDROGEN TO HIGH PRESSURE SYNTHESIS
FINAL FERMENTORS 60,000 — 500,000 GALLONS
H₂, CO₂
SCRUBBER
CO₂
COMPRESSOR
DRY ICE
BEER WELL
STILLAGE
TRIPLE EFFECT EVAPORATOR
CONTINUOUS STILLS
CRUDE SPIRITS (HIGH WINES)
SPRAY OR DRUM DRYERS
FRACTIONATING COLUMNS
DRIED BUTYL STILLAGE
ACETONE 30 PARTS)
ETHYL ALCOHOL (10 PARTS)
BUTYL ALCOHOL (60 PARTS)

8. Flow diagram of typical modern acetone-butanol fermentation using starch
ıcts. [*Courtesy of S. C. Beesch, Appl. Microbiol.,* **1**(2): 85 (1953).]

**aw Materials.** Although many kinds of sugar-containing raw mate- may be used by the saccharolytic acetone-butanol bacteria, the red ones are invert ("high-test") and blackstrap molasses. Sucrose, se, beet molasses, citrus molasses, hydrol, sulfite liquors, and sac- fied starches may also be used as sources of sugars.

rogen is supplied through the use of degraded protein nitrogen (such lypeptides, amino acids, and ammonia and ammonium salts) and the f corn-steep liquor, yeast water, or distillation slops. Although some nia may be added to the mash initially to bring the pH to 5.7 to 6.7, of it is added after the fermentation has proceeded for about 16 hr.

TABLE 79. RATIOS OF SOLVENTS PRODUCED BY VARIOUS SACCHAROLYTIC BACTERIA *

| U.S. patent no. | Names of bacteria | Substrate | Solvent ratios, % | | | |
|---|---|---|---|---|---|---|
| | | | Butyl alcohol | Ethyl alcohol | Acetone | Isopropyl alcohol |
| 1,725,083 | *Bacillus saccharobutylicum—beta* | Inverted molasses and $CaCO_3$ | 75 | ..... | 3 | 35 |
| 1,908,361 | *Clostridium saccharobutylicum—gamma* | Blackstrap molasses and $CaCO_3$ | 65–80 | ..... | 18–34 | 1–2 |
| 1,922,921 | *Cl. saccharobutyl-acetonicum* | Blackstrap molasses, corn gluten, and $(NH_4)_2SO_4$ | 64 | ..... | 36 | |
| 2,017,572 | *Cl. viscifaciens* | Inverted molasses and $CaCO_3$ | 66 | ..... | 3 | 31 |
| 2,050,219 | *Cl. saccharoacetobutylicum—beta and gamma* | Cane molasses and degraded protein, such as ammonia, steep water, or distillery slop | 68–73 | 1–3 | 26–32 | |
| 2,063,448 | *Cl. propyl butylicum* | Inverted molasses, $NH_3$, and $CaCO_3$ | 69–70 | ..... | 4–17 | 14–28 (mixture isopropyl and ethyl) |
| 2,073,125 | *Cl. invertoacetobutylicum* | Louisiana molasses (inverted) and ammonium salts or alkalies | 66–70 | 2–3 | 27–31 | |
| 2,089,522 | *Cl. saccharoacetobutylicum* | Louisiana molasses, $(NH_4)_2SO_4$, and $CaCO_3$ | 68–73 | 1–3 | 26–32 | |
| 2,096,377 | *Cl. saccharobutyl-isopropyl-acetonicum* | Inverted molasses and degraded protein | Low pH, 60–70; High pH | ..... | 30–38<br>2–20 | Trace 10<br>10–30 |

|  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
|  |  | and gluten meal |  |  |  |  |
| 2,113,472 | *B. tetryl* |  | 74 | 6 | 20 |  |
| 2,132,039 | *Cl. propyl butylicum—alpha* | Inverted molasses, $(NH_4)_2SO_4$, $CaCO_3$, $K_2HPO_4$, and $MgSO_4$ | 65–70 | 3–4 | 5–10 | 16–20 |
| 2,139,108 | *Cl. saccharobutyl-acetonicum-liquefaciens —gamma* and *delta* | Blackstrap molasses, $(NH_4)_2SO_4$, $CaCO_3$, and $P_2O_5$ | 58–74 | 2–6 | 24–36 |  |
| 2,139,111 | *Cl. saccharobutyl-acetonicum-liquefaciens —gamma* and *delta* | Cuban molasses, $(NH_4)_2SO_4$, $CaCO_3$, and $P_2O_5$ | 60–69 | 3–4.5 | 26–35 |  |
| 2,147,487 | *B. butacone* | Blackstrap molasses and animal and vegetable protein | 65 | ..... | 28 |  |
| 2,169,246 | *Cl. celerifactor* | Inverted molasses, ammonia, and $CaCO_3$ | 60 | 2 | 38 |  |
| 2,195,629 | *Cl. granulobacter acetobutylicum* | Molasses, corn gluten, ammonium salts, and $CaCO_3$ | 60–75 | 1–10 | 25–30 |  |
| 2,219,426 | *Cl. saccharobutyl-isopropyl-acetonicum —beta* | Cane and beet molasses, $(NH_4)_2SO_4$, and $CaCO_3$ | 60–85 | ..... | 15–40 | 0.1–4.0 |
| 2,398,837 | *Cl. madisonii* | Cuban blackstrap, $NH_4OH$, $(NH_4)_2SO_4$, and $CaCO_3$ | 75–76 | 4–6 | 17–20 |  |
| 2,420,998 | *Cl. amylosaccharobutyl-propylicum* | Invert molasses, $(NH_4)_2SO_4$, $CaCO_3$, and $P_2O_5$, or $NH_4OH$ and $P_2O_5$ | 65–72 | Trace | 2–4 | 26–32 |
| 2,439,791 | *Cl. saccharoacetoperbutylicum* | Invert molasses, $(NH_4)_2SO_4$, $CaCO_3$, and $P_2O_5$, or $NH_4OH$ and $P_2O_5$ | 69–76 | 2–7 | 18–25 |  |

---

* Courtesy of S. C. Beesch, *Ind. Eng. Chem.*, **44**: 1677 (1952). Copyright 1952 by the American Chemical Society and reprinted by permission of the copyright owners.

When ammonium salts are used, the quantity employed is 4 to 6 per based on the weight of the sugar, of ammonium sulfate or its equiv Calcium carbonate is used to maintain the pH when ammonium salt a component of the mash. The usual amount of calcium carbonate i 7 per cent of the weight of the sugar.

Ethyl grain, butyl grain, and molasses stillages may be used to the fermentation mash and serve as a source of nutrients. The u stillage results in better yields. However, additional amounts of ac may be produced with a change in the solvents ratio.

If the mash is deficient in respect to phosphorus, this element m supplied through the use of calcium acid phosphate, monoammo phosphate, superphosphate, or other soluble phosphate, in such co tration as to provide 0.2 to 0.4 per cent of phosphorus pentoxide o basis of the weight of carbohydrate used. For example, 0.3 per ce superphosphate or monoammonium phosphate, on the weight o sugar, is added to a mash containing invert molasses.

**Sterilization.** The mash, the fermentation tanks, the lines, an equipment which comes into contact with mash and bacteria befor completion of fermentation are sterilized with steam. Mashes are tinuously sterilized, as in the patented process of Carnarius (1 Initially the mash is heated to 225°F. and circulated for a period of in a filled system. Then it is withdrawn continuously, while fresh ma is fed into the system. The temperature of the sterilized mash i duced to 88 to 90°F. by being pumped through double-pipe co The cooled mash is pumped to the sterile fermentors through ster lines.

**Preparation of the Inoculum.** As described by Beesch (1952) culture for seeding the main molasses fermentation mash may be bui as follows: Spores of the selected species are added to sterile potato cose medium in a test tube. The latter is placed in boiling wate 90 sec., then cooled immediately to 87°F. and incubated for 20 to 2 at this temperature. This culture is used to inoculate 600 ml. of a s medium containing 4 per cent sugar (in the form of invert or high molasses), 5 per cent ammonium sulfate, 6 per cent calcium carbo and 0.2 per cent phosphorus pentoxide (in the form of superphosp (the quantities of chemicals used are based on the weight of the sug the molasses). After incubation for 20 to 24 hr., the culture is used to 3,000 ml. of sterile medium of the same composition contained 4,000-ml. Erlenmeyer flask (about 3 per cent of inoculum is used). a period of 20 to 24 hr., this culture is used to inoculate 4,000 gal. molasses mash containing 6 per cent sugar and the same percenta chemicals as employed in the laboratory stages, provided that no in tion of contamination has been observed as a result of aerobic pla made from preceding mashes and that the fermentation appears t normal in all respects. The contents of the tank are mixed mechani for 5 min. after the addition of the culture. An atmosphere of s fermentation gases is maintained at a pressure of 15 lb. over the m in the tank for the purposes of supplying anaerobic conditions and

ing contaminants from entering. The temperature is held at 87°F.
ugh the circulation of water or steam in the jacket of the tank.

uring the ensuing fermentation and development of bacterial cells,
ful observation is made of the Brix readings, the pH, and the titrable
ity of the mash. After the titrable acidity reaches a maximum and
s to decline, the culture is used to seed the main fermentation mash,
h is contained in tanks ranging in size from 60,000 to 500,000 gal.

**lling the Fermentor.** The sterilized mash is admitted to a sterilized
entor through sterilized lines. While the mash is being run into the
, the inoculum is also added. In the more common method of filling,
noculum is added at the beginning and all of the mash is added to
ermentor at one time. In the other method, all of the inoculum and a
on of the mash are added to the fermentor initially and the rest of
nash is added after a few hours. In this "deferred-filling method,"
ulture multiplies several times before the rest of the mash is added,
erating the final fermentation and tending to reduce the growth of
minants.

**rmentation Conditions.** The optimum temperature for this fer-
ation is 87°F. The pH may vary from 5 to 7; however, the initial
s usually 5.5 to 6.5, and the final pH is normally 5.2 to 6.2. Since the
entation is anaerobic, it may be advantageous to maintain sterile
n dioxide at a pressure until the bacteria have had the opportunity
ilding up a pressure of their own.

ien ammonia is added to a fermenting mash, it is usually added in
ments within a period of 18 to 24 hr. Table 80 shows the rate of
ion of ammonium hydroxide to a mash containing 200,000 gal.

**ow Sheet.** Figure 39 shows a typical and modern acetone-butanol
ntation in a flow diagram.

**elds.** Data concerning the yields of the various end products of the
ntation of blackstrap molasses are shown in Table 81.

## PRODUCTION FROM OTHER SUBSTANCES

n and molasses have been the principal raw materials used for the
ction of butanol and acetone by fermentation in this country because
ir availability, ease of handling, and relatively low price. However,
are other carbohydrate sources that offer potentialities. Some of
will be mentioned briefly.

**oduction from Waste Sulfite Liquor.** The production of acetone
utanol from waste sulfite liquors has been studied by Wiley and
ates (1941). They found that good yields of the solvents were
ied when the sulfite liquor was prepared by precipitating the sulfur
e with calcium hydroxide at a pH of 10 as calcium sulfite, by pre-
ting the lignin by adjusting the pH to 11.5 with additional calcium
xide, by precipitating the excess calcium with 1 per cent by weight
ium sulfate before neutralizing, and by neutralizing the liquor to
f 5.8 with sulfuric acid. The precipitates formed during the various
ients were removed. The following nutrients were found to be satis-

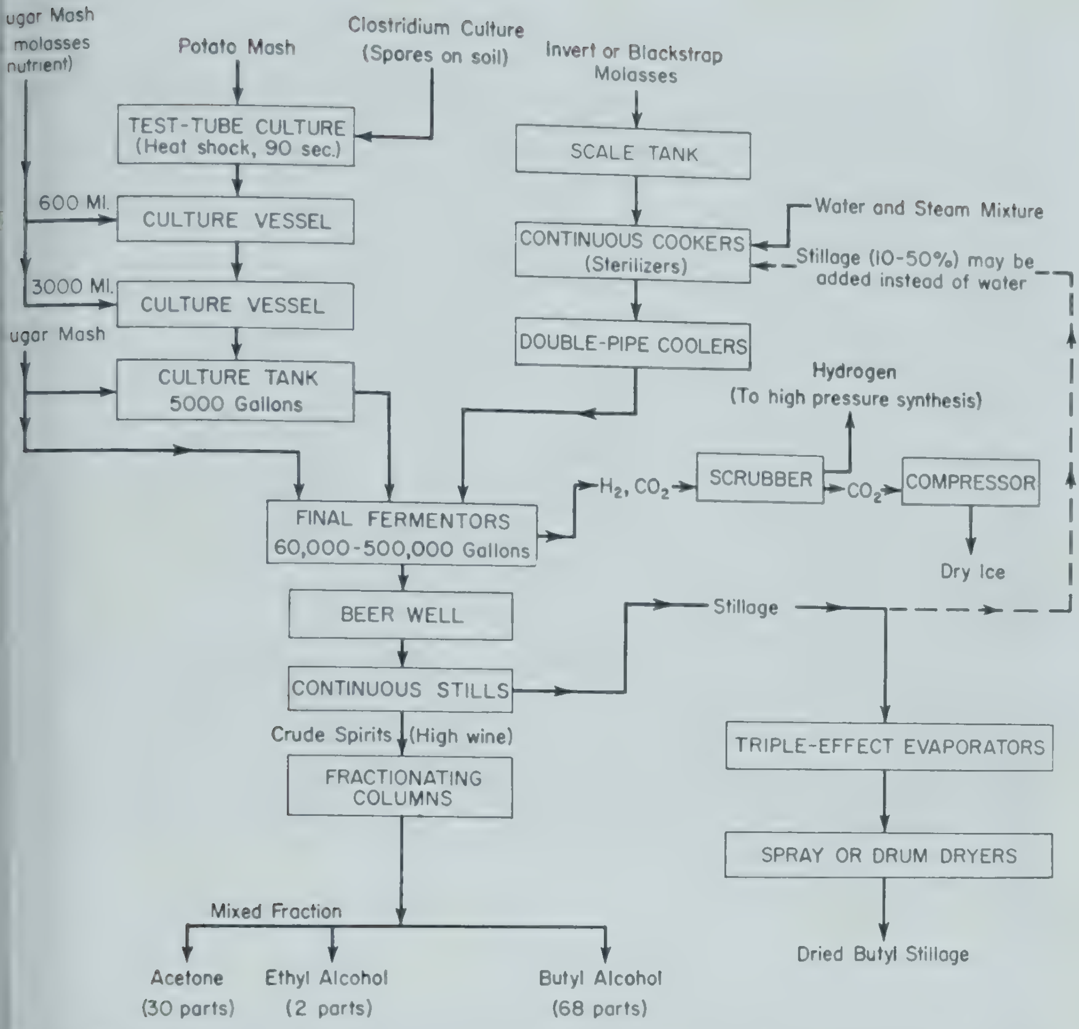
TABLE 80. ACETONE-BUTANOL FERMENTATION OF BLACKSTRAP MOLASSES

| Age, hr. | pH | ° Brix | Gas rate, units | NH$_4$OH add (28%), gal |
|---|---|---|---|---|
| 1 | .... | ... | .. | 70 |
| 2 | 5.55 | 8.7 | | |
| 3 | | | | |
| 4 | 5.45 | 8.6 | | |
| 5 | | | | |
| 6 | 5.45 | 8.6 | 2 | |
| 7 | | | 8 | |
| 8 | 5.40 | 8.5 | 10 | |
| 9 | | | 11 | |
| 10 | 5.25 | 8.0 | 12 | 30 |
| 11 | | | 13 | |
| 12 | 5.15 | 8.0 | 14 | 30 |
| 13 | | | 15 | 40 |
| 14 | 5.30 | 7.8 | 15 | 50 |
| 15 | | | 15 | 50 |
| 16 | 5.20 | 7.8 | 15 | |
| 17 | | | 16 | |
| 18 | 5.30 | 7.5 | 17 | 60 |
| 19 | | | 18 | 70 |
| 20 | 5.35 | 7.3 | 19 | 80 |
| 22 | 5.35 | 6.9 | 20 | 110 |
| 24 | 5.30 | 6.9 | 20 | 160 |
| 26 | 5.65 | 6.5 | 21 | |
| 28 | 5.65 | 6.5 | 21 | |
| 30 | 5.55 | 6.4 | 22 | |
| 32 | 5.55 | | | |
| 34 | 5.30 | 6.1 | | |
| 36 | 5.35 | 5.6 | | |
| 38 | 5.35 | 5.2 | | |
| 40 | 5.35 | 4.6 | | |
| 42 | 5.35 | 4.1 | | |
| 44 | 5.40 | 3.4 | | |
| 46 | 5.35 | 3.3 | | |
| 48 | 5.40 | 3.0 | | |

Fermentation data

| | |
|---|---|
| Volume of mash | 200,000 gal. |
| Concentration of sugar | 6 % |
| Culture (*Clostridium saccharoacetoperbutylicum*) | 2 % |
| Total NH$_4$OH (28%) | 730 gal. |
| Total mixed solvents | 18.57 g./l. |
| Acetone | 20.93 % |
| *n*-Butyl alcohol | 74.70 % |
| Ethyl alcohol | 4.37 % |
| Yield of total solvents from sugar | 31.0 % |
| Residual sugar | 0.45 g./100 m |
| Residual ammonia | 11 p.p.m. |
| Fermentation temperature | 87°F. |

* Courtesy of S. C. Beesch, *Ind. Eng. Chem.*, **44**(7): 1677 (1952). Copyrigh by the American Chemical Society and reprinted by permission of the co owners.


ugar Mash
molasses
nutrient)
Potato Mash
Clostridium Culture
(Spores on soil)
Invert or Blackstrap Molasses
TEST-TUBE CULTURE
(Heat shock, 90 sec.)
SCALE TANK
600 Ml.
CULTURE VESSEL
Water and Steam Mixture
CONTINUOUS COOKERS
(Sterilizers)
Stillage (10-50%) may be added instead of water
3000 Ml.
CULTURE VESSEL
ugar Mash
DOUBLE-PIPE COOLERS
CULTURE TANK
5000 Gallons
Hydrogen
(To high pressure synthesis)
$H_2$, $CO_2$
SCRUBBER
$CO_2$
COMPRESSOR
FINAL FERMENTORS
60,000-500,000 Gallons
Dry Ice
BEER WELL
Stillage
CONTINUOUS STILLS
Crude Spirits
(High wine)
TRIPLE-EFFECT EVAPORATORS
FRACTIONATING COLUMNS
SPRAY OR DRUM DRYERS
Mixed Fraction
Dried Butyl Stillage
Acetone
(30 parts)
Ethyl Alcohol
(2 parts)
Butyl Alcohol
(68 parts)

39. Flow diagram of typical modern acetone-butanol fermentation using sugar ets. [*Courtesy of S. C. Beesch, Ind. Eng. Chem.*, **44**(7): 1677 (1952). *Copyright by the American Chemical Society and reprinted by permission of the copyright r.*]

TABLE 81. MATERIAL BALANCE IN ACETONE-BUTANOL FERMENTATION OF BLACKSTRAP MOLASSES *

| Starting material: 100 lb. blackstrap molasses containing: | lb. |
|---|---|
| Total solids | 81.5 |
| Sucrose and invert sugar | 57.0 |
| Protein | 3.1 |
| Ash | 6.2 |
| Fermentation data: Culture of saccharolytic bacteria used with 1% $NH_3$ added in form of ammonium hydroxide | |
| Yields: | |
| Butyl alcohol | 11.5 |
| Acetone | 4.9 |
| Ethyl alcohol | 0.5 |
| Carbon dioxide | 32.1 |
| Hydrogen | 0.8 |
| Dry feed containing (6 lb. protein, 6 lb. ash) | 28.6 |

ourtesy of S. C. Beesch, *Ind. Eng. Chem.*, **44**: 1677 (1952). Copyright 1952 by the ican Chemical Society and reprinted by permission of the copyright owners.

factory: 0.05 per cent diammonium hydrogen phosphate $(NH_4)_2$ 0.10 per cent molasses, and 0.10 per cent calcium carbonate. *Clost butylicum* (Fitz strain) was found to be the most suitable org From 70 to 80 per cent of the total reducing substances found in t fite liquor were fermentable, and from 25 to 30 per cent of the were fermented to neutral solvents in the following proportions: 7 of butanol, 20 parts of acetone, and 5 parts of ethanol.

**Production from Wood Sugar.** Leonard, Peterson, and Ritter found that 24 to 38 per cent of the wood sugars fermented were co to neutral solvents by *Cl. butylicum* (No. 39 of the University o consin collection). The hydrolyzates were distilled to remove the f and neutralized to a pH of 6.5 with lime. It was found that suga tions produced by very mild or very vigorous conditions of hyd were not easily fermented. Sugar concentrations up to 3 per cen completely utilized.

**Production from Xylose Saccharification Liquors from Cor** Butyl alcohol may be produced in satisfactory yields from the xylo charification liquors obtained from corncobs in the first stage of th stage process described by Dunning and Lathrop (1945), after tre with powdered iron or activated carbon, according to a report m Langlykke, Van Lanen, and Fraser (1948).

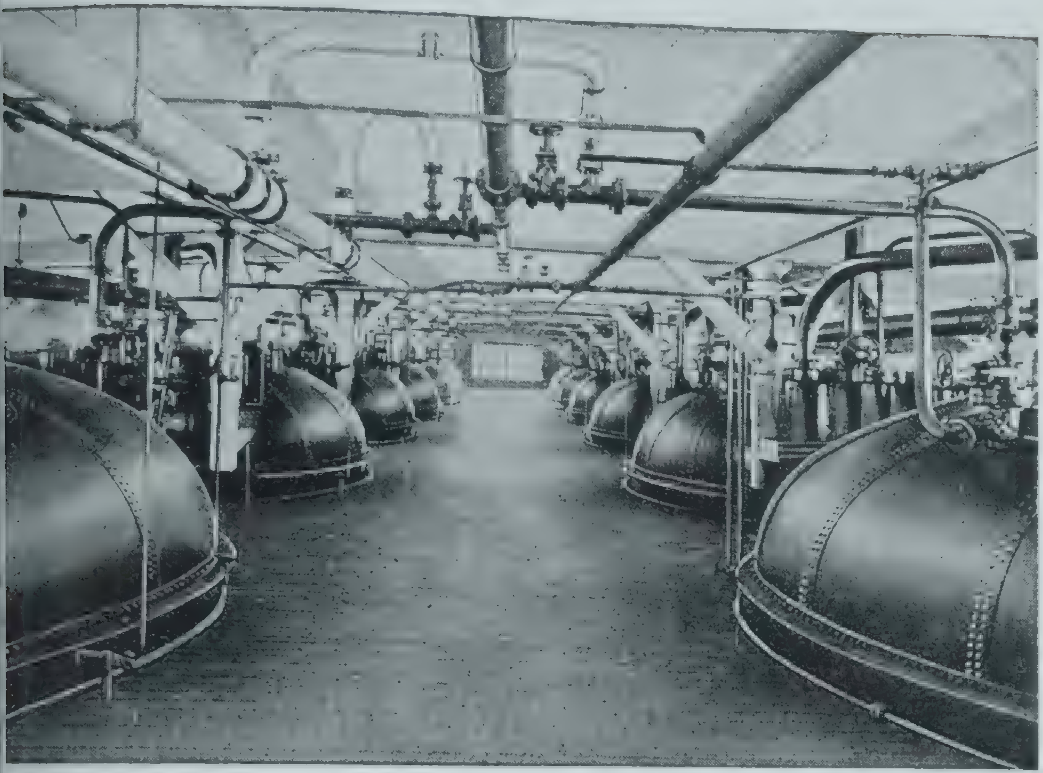
The following nutrients were used in the media: 0.2 per cent cor liquor (dry basis), 0.5 per cent calcium carbonate, 0.3 per cent amm sulfate, and 0.06 per cent dibasic ammonium phosphate. The sugar was usually diluted to produce a final concentration of 5 to 6 per total sugar. The media were adjusted to pH 6.5 prior to sterili Incubation was at 37°C.

Satisfactory fermentation of saccharification liquors was obtaine they were treated by the addition of 0.5 per cent of reduced iron powder. The use of iron filings ground to pass a 325-mesh scree suitable as a substitute for reduced iron. The iron removed coppe the hydrolyzates and also lowered their oxidation-reduction pote Copper in concentrations greater than 15 p.p.m. was found to be inh to the butanol organism [culture A-14 (NRRL B-594) of M collection at the University of Wisconsin] under the conditi fermentation.

The treatment of the hydrolyzate with 15 per cent or more act carbon (on the basis of the sugar) was also a satisfactory means f ducing good yields.

Yields of 30.6 per cent of solvent were obtained on the avera which 61.7 per cent was butanol, 31.8 per cent was acetone, and cent was ethanol.

**Production from Jerusalem Artichokes.** The production of b and acetone from Jerusalem artichokes has been studied by We Fulmer, and Underkofler (1941). The ground chips were dilute water to yield a reducing sugar content of 5 per cent. The sus was mildly hydrolyzed for 1 hr. at 80°C. after adjustment of t to 1.75 with hydrochloric acid or to 1.50 with sulfuric acid. The hy



0. Upper level of 50,000-gal. fermentation tanks. (*Courtesy of G. O. Lines, nercial Solvents Corp.*)

. Lower level of 50,000-gal. fermentation tanks. (*Courtesy of G. O. Lines, ercial Solvents Corp.*)

zate was neutralized to a pH of 5.5 to 6 with sodium hydroxide. found necessary to add corn meal, soybean meal, or other nutrient hydrolyzate in order to obtain maximum yields with *Cl. acetobu*

**Production from Cassava.** Cassava only is not a good subst acetone-butanol production, according to Banzon (1941). Howev yields could be obtained from a mixture of 20 per cent or more and 80 per cent or less of cassava. Likewise, yields of solvents com to those obtained from corn were secured when about 5 per cent of powder, corn gluten meal, and soybean flour were used as constit the mash.

## GENERAL DETAILS OF THE PROCESS

**Microorganisms.** Data concerning the bacteria used for the tion of acetone and butanol have already been given. Additiona will now be presented.

There is, of course, a fairly large number of organisms, some no forming, which will produce butanol, but these bacteria would be factory for commercial practice for various reasons. Some are path some will produce only small quantities or traces of solvents, so slow fermentors, some will not attack starch, some are aerobic, an lack vigorous cultural characteristics.

A cultural study of the acetone–butyl alcohol organism embodyi factors as reaction of the medium, temperature, reduction of dy fermentability of carbohydrates was carried out by McCoy, Fred son, and Hastings.

McCoy and her associates (1930) have classified the motile and forming butyric anaerobes of fermentation as a nonpathogenic su of the genus *Clostridium,* which yields larger amounts of butyric neutral products than the pathogenic clostridia, gives the gr reaction, and is catalase negative. The butyric anaerobes were into two general groups:

Group 1. Acid end products, chiefly butyric and acetic acids— butyric anaerobes.

- Subtype *A*. *Cl. pasteurianum* type—nonstarch-fermenting ba
- Subtype *B*. *Bacillus saccharobutyricus* type—starch-fermenti tridia and occasionally plectridia
- Subtype *C*. Starch-fermenting plectridia

Group 2. Butyric and acetic acids as intermediate products, f by neutral products (alcohols, or alcohol plus acetone)—the organisms of industry.

**Culture Methods.** The continued transfer of a seed culture usual media results in the culture becoming sluggish, with a corres decreased yield of solvents. This observation has been made in ently by several different workers.

It has been shown that the most prodigious producers of solv those cultures whose spores are in general the most heat resistant.

"Heat shocking" is a method wherein the vegetative cells a er spores of a culture are destroyed. By subjecting a culture of the nism, in which the development of spores has been favored, to a erature of 100°C. for 1 to 2 min., heat shocking is effected.

must be borne in mind that the severity of the treatment bears a t relation to the temperature employed, the size and the nature of the containing the seed culture, and the characteristics of the medium. e is to use thin-walled wafer tubing and a very small amount of the re, it is obvious that neither the time of shocking nor the tempera- should be excessive, since even the resistant spores may be destroyed. eyer and Rettger "pasteurized" their butanol cultures in capillary s, which were 5 cm. in length. The tubes were filled with saline sus- ions of the spores and plunged into water at 100°C. An exposure 5 sec. to this temperature was the limit of the tolerance of the spores. capillary tubes were cooled quickly after the heat-treatment.

lternate heat shocking, or pasteurizing, and successive subculturing commonly used to activate a culture. The medium containing the culture is allowed to stand at room temperature for a few days to urage sporulation. New medium is inoculated from the spore-con- ing culture and heat-shocked. The surviving spores are permitted to minate under favorable conditions, and subcultures are made succes- ly at daily intervals for 4 to 7 days. At the end of this time the ure is again permitted to stand at room temperature to encourage re formation. The cycle is then repeated—heat-treatment, repeated culturing, rest for sporulation, heat shocking, and so on.

eizmann advocated heat treating a culture 100 to 150 times in order mprove its fermenting ability.

nderkofler and his coworkers (1937) have shown that it is impossible btain good sporulation from sugar media when using ordinary culture es in the usual manner. They have demonstrated that sporulation ld be successfully induced, however, provided the culture-containing lium was poured aseptically into a sterile flask of such size that the lium was exposed in a thin layer to air or was dried on sterile soil.

n a study of six different strains of *Cl. acetobutylicum* (Weizmann), yer and Rettger (1927) brought out several facts of major importance. rage of spores for a period of over 6 months apparently decreased their ver of producing solvents. It was possible, however, to rejuvenate the tures by alternate pasteurization and subculturing, for pasteurization troyed the vegetative forms and the weaker spores. The most vigorous ains for fermentation appeared to be the most active sporeformers.

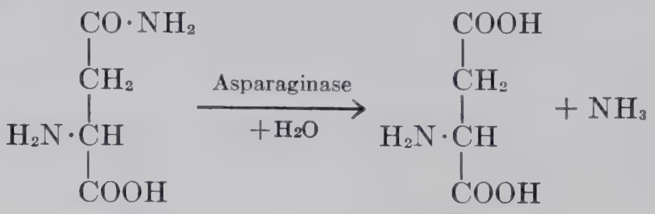
**Raw Materials.** A rather wide variety of raw materials may be used supply the carbohydrates and nutrient substances required to ensure a isfactory fermentation. Starches rendered soluble by preliminary treat- nt, hydrolyzed starches, disaccharides, hexoses, pentoses, molasses, and gar sirups have been used under different conditions. Corn is, of course, readily available and easily fermented source of raw material in this untry and elsewhere. Rice, jawari, bajra, and tapioca starches; peanut d oat hulls; corncobs; horse chestnuts; arabinose; and xylose have been rmented by butanol organisms with varying degrees of success. Underkofler and others have shown experimentally that as much as 80 pe of corn meal may be replaced by sucrose or glucose without sacri high yields. Xylose may replace corn meal to the extent of 40 per Thus it is possible to ferment along with corn meal materials whic themselves, would produce small yields or be fermented with some culty. This is a sound practice from the point of view of conserv and economics.

**Vitamins.** Biotin and *p*-aminobenzoic acid are necessary fo growth of some acetone-butanol organisms. These requirements ma satisfied by the use of yeast extract or other raw materials which co the essential vitamins.

**Substances Stimulating the Production of Butanol by Ce Butyric Acid Bacteria.** In 1934, Tatum, Peterson, and Fred repo the presence of an unknown substances in potatoes, yams, cabbage tuce, alfalfa, navy beans, soybeans, malt sprouts, and wheat midd that caused an increased fermentation of starch and a marked incr in the yield of butanol through the action of certain butyric acid bact No changes were effected in the quantities of ethyl alcohol and ace produced. Barley, corn, oats, and rice were poor sources of the sti lating substance and sometimes were found to contain none of it.

Later, Tatum and his associates (1934) identified L-aspara $(\mathrm{H_2NOC\cdot CH_2\cdot CH(NH_2)\cdot COOH})$ as the substance producing the sti latory action. Further research disclosed that L-aspartic $(\mathrm{HOOC\cdot CH_2\cdot C}$ $(\mathrm{NH_2})\cdot\mathrm{COOH})$ and D-glutamic $(\mathrm{HOOC\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COC}$ acids, which are dicarboxylic amino acids, produced effects equivalent those of L-asparagine when these acids were supplemented with mo equivalents of ammonium sulfate. The optimum concentration of th stimulants was 60 mg. per 100 ml. of 4 per cent corn-meal mash, altho lower concentrations produced stimulation.

Asparagine may be hydrolyzed by the enzyme asparaginase to yi aspartic acid and ammonia.



$$\begin{array}{c}\mathrm{CO\cdot NH_2}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{H_2N\cdot CH}\\ |\\ \mathrm{COOH}\end{array} \xrightarrow[+\mathrm{H_2O}]{\text{Asparaginase}} \begin{array}{c}\mathrm{COOH}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{H_2N\cdot CH}\\ |\\ \mathrm{COOH}\end{array} + \mathrm{NH_3}$$

Asparagine Aspartic acid

Asparaginase is secreted by some yeasts and bacteria and has an op mum pH of 8.

Ammonium malate $(\mathrm{H_4NOOC\cdot CH_2\cdot CHOH\cdot COONH_4})$ and amn nium succinate $(\mathrm{H_4NOOC\cdot CH_2\cdot CH_2\cdot COONH_4})$ were likewise found stimulate the fermentation of starch by certain, but not all, butyric a bacteria and to cause increases in the yields of butanol, but these su stances were less effective than L-asparagine.

**Oxygen Relationship.** Since the organisms best suited to the prod tion of acetone and butanol are anaerobic in nature, the highest yie will be obtained when anaerobiosis is maintained.

I. Growth may be obtained in corn mashes between a pH of 4.7 and the butanol organisms, but there is a low production of solvents at of the extremes. The pH range of 5 to 7 is in most cases satis- ry.

e assertion that a given concentration of hydrogen ions is completely itory at all times or is capable of producing any given degree of ition, is not justified, according to Wynne (1931), who carried out riments upon the inhibition of the acetone-butanol fermentation by :. The inhibition of fermentation was in the following order: nonylic iprylic > heptylic > formic > isocaproic = caproic > valeric = iso- ic > isobutyric = butyric ≧ propionic = acetic. This order is simi- o that of the speed of penetration of the acids into the living cells. pH necessary for pyruvic acid to inhibit is 3.2, which is lower than nost acids.

**ffect of Calcium Carbonate.** In general, the addition of calcium onate to the acetone-butanol fermentation medium causes a decrease he yields of acetone and butanol in proportion to the amount of ium carbonate added. The volatile acid content, butyric and acetic s, principally, is increased, while alcohol formation is suppressed.

**oncentration of Raw Material.** The exact concentration of the ohydrate-containing substance to be used in a given fermentation t be determined by experimentation, unless facts concerning the fer- itation organism are already known. Mashes containing 3 to 10 per : concentrations of corn meal are readily fermented with high yields.

**acterial Contaminants.** The most serious contaminants in the tone-butanol fermentation are the lactic acid organisms. Lactic acid teria grow readily at the temperature of the butanol fermentation under anaerobic conditions. They utilize the substrate and at the e time produce a pH unfavorable for butanol production. The high- l-forming bacteria (Fred et al., 1926), such as *Lactobacillus leich- mii*, are most injurious. Organisms such as *L. mannitopoeum* are like- very undesirable contaminants. *B. volutans* n. sp. Fleming, Thaysen iaysen, 1921), a most serious contaminant, is a nonsporeforming anism that produces large amounts of lactic acid, traces of butyric acetic acids, but no gas or alcohols. It is a Gram-positive organism, sessing volutin granules that may be stained by methylene blue to a p purple. This organism may be destroyed by heating it for 5 min. 55°C. *Streptococcus lactis* also has been known to cause infections, but does not usually produce serious trouble. Fermentations continue to npletion unless the organisms are present in large numbers in the mash fore the butanol organisms have had opportunity to develop.

The presence of *B. globigii* in a butanol fermentation is a type of asso- tion that produces no apparent ill effect on the yield by *B. granulo- cter* (Fred et al., 1925).

Sporeformers of the *B. mesentericus* group may produce a red pigment corn mashes but otherwise produce no apparent harm.

**Methods of Detecting Contamination.** Various methods have been ed to detect contamination in the fermenting mash. Observation of variations in gas evolution or in the titrable-acidity curve and th the microscope are the most common methods.

Perhaps the most sensitive indicator of contamination is gas ev One accustomed to observing normal fermentations can readily as irregularities in the evolution of gas. A marked reduction in the of gas evolved at a time when the rate of evolution should be inc may be taken as an indication of contamination. Gas evolution ma cease.

By plotting the curve for titrable acidity, one has available ac information concerning the progress of the fermentation. Any incr acidity to abnormal amounts can be readily ascertained by an exa tion of the acid curve. After rising to a peak in 13 to 17 hr., the n curve for titrable acidity drops at about the same rate to a ne point. In case the mash is contaminated by lactic acid organism curve for titrable acidity continues to rise after the normal max instead of returning to the new low point. In some industrial pla practice was made to inoculate large mashes from starters only aft curve for titrable acidity had commenced to fall. (The reader s realize that variations in the nature of the curve for titrable acidity s times occur even though there is an absence of infection.)

The microscope has served as a useful tool in detecting contamina yet it may fail to show contamination when variation in either gas lution or titrable acidity may strongly indicate the presence of infe organisms. The microscope is best used in conjunction with one o foregoing methods rather than alone.

Marked changes in the curve for pH would indicate unusual condi in the fermenting mash. Changes in pH do not, however, furnish e as sensitive or as accurate signs of an abnormal fermentation a abnormal variations in gas evolutions and in titrable-acidity curves.

## END PRODUCTS OF FERMENTATION

The normal end products of the acetone-butanol fermentation ar neutral solvents (acetone, butanol, and ethanol), the acids (formic, a and butyric), the gases (carbon dioxide and hydrogen), acetylme carbinol, and yellow oil.

Data have already been presented relating to the ratios of the ne solvents. Additional data are given in Table 82.

From 3 lb. of starch, 1 lb. of mixed solvents may be obtained *Clostridium acetobutylicum* is used as the fermentation organism.

At the Royal Naval Cordite Factory at Holton Heath, England lb. of *n*-butanol, 70 lb. of acetone, 407 lb. of carbon dioxide, 11 l hydrogen, and 12 lb. of residual acid were obtained from 1,000 l maize, which contained 650 lb. of starch (Reilly et al., 1920).

The gases produced during fermentation weigh over 1.5 times as r as the neutral solvents formed. Were these gases allowed to go to w as they were at one time, the losses would be great.

**Acetylmethylcarbinol.** In 1927, Wilson and his associates sh that acetylmethylcarbinol was produced in the acetone-butanol ferme

ABLE 82. AVERAGE SOLVENT RATIO FOR THE ACETONE-BUTANOL FERMENTATION OF SUGARS *

| vestigators | Sugar | No. of fermentations | Average solvent ratio | | |
|---|---|---|---|---|---|
| | | | Butanol | Acetone | Ethanol |
| son, Fred, and midt † | Glucose | 2 | 59 | 31 | 10 |
| | Xylose | 3 | 61 | 29 | 10 |
| | Arabinose | 2 | 47 | 43 | 10 |
| on, Peterson, Fred | Glucose | 18 | 60 | 28 | 12 |
| | Arabinose | 4 | 48 | 39 | 13 |

. A. Underkofler and J. E. Hunter, Jr., *Ind. Eng. Chem.*, 30: 480 (1938). Copy-
·1938 by the American Chemical Society and reprinted by permission of the
ight owner.
tios calculated from the data, assuming 10 per cent ethanol.

by *Cl. acetobutylicum* (Weizmann's bacillus) as a regular end prod-
Acetylmethylcarbinol was formed concurrently with acetic and
ric acids. Pyruvic acid when added to a fermenting mash was fer-
ted to acetic acid, acetone, and acetylmethylcarbinol chiefly (Peter-
and Johnson, 1933). The addition of phosphates to a mash increased
production of acetylmethylcarbinol, while added proteins decreased
yield. Ordinarily 300 to 400 mg. per liter of acetylmethylcarbinol is
luced in the butanol fermentation.

**ellow Oil.** In the butanol fermentation, so-called "yellow oil" makes
).5 to 1 per cent of the total yield of solvents. Marvel and Broderick
25) showed that the high-boiling yellow oil was a complex mixture
-butyl alcohol, active amyl alcohol, isoamyl alcohol, *n*-hexyl alcohol,
the *n*-butyric, caprylic, and capric esters of these alcohols.

## BIOCHEMISTRY OF THE FERMENTATION

eakman (1920) divides the acetone-butanol fermentation into three
es, the length of each phase being governed by the curve for titrable
ity. During the first phase, the titrable acidity increases rapidly to a
imum, usually in 13 to 17 hr. The butanol organisms reproduce very
dly during this period, for it is their enzymic activity that deter-
es the progress of the fermentation. Acetic and butyric acids are
duced in varying quantities. Both hydrogen and carbon dioxide gases
produced in large amounts, the curve for total gas evolution following
titrable-acidity curve in general proportions but with a time lag.
ere is a drop in pH, which then tends to remain at a fairly constant
l throughout the rest of the fermentation, owing to the presence of
fers produced through the hydrolysis of the corn or other proteins.
he quantity of titrable acidity drops sharply during the second phase
fermentation to a value that is equal to approximately 50 per cent of
maximum. Coincidental with the drop in the quantity of titrable
dity, there is a rapid conversion of the acids to their corresponding

solvents. Butyric acid is reduced to butanol, and acetic acid is to acetone. The ratio of acetic to butyric acid varies during this Butyric acid disappears from the fermentation mash more rapid acetic acid. The rate of gas evolution increases quickly to a ma as the titrable acidity drops from the peak. There is then a diminution in the rate of gas evolution until the end of the ferme

The titrable acidity slowly increases in quantity during th phase of the fermentation. There is a drop in the rate of solvent tion until the fermentation ceases. The relative proportions of ace butyric acids continue to vary until at the end of the fermentatio is a greater amount of acetic acid.

Peterson and Fred have carried out extensive research on the ology and biochemistry of the acetone-butanol fermentation by *tridium acetobutylicum.* Figures 42 to 47 are reproduced (throug courtesy) from their excellent paper. The data from which the were constructed were determined by periodic analyses of the ferm corn mashes, which at the start were of 6 per cent concentration. shown in Figs. 42 to 45 were obtained in the first experiment of Pe and Fred, those of Fig. 46 in the second experiment, and those of in the third experiment. A study of these figures will yield much va information.

Only small amounts of peptides or amino acids are formed from mashes during the first 12 hr. of the fermentation and not much the first 24 hr., according to Peterson and his associates. The amin and peptide content of the mash increases rapidly during the next however.

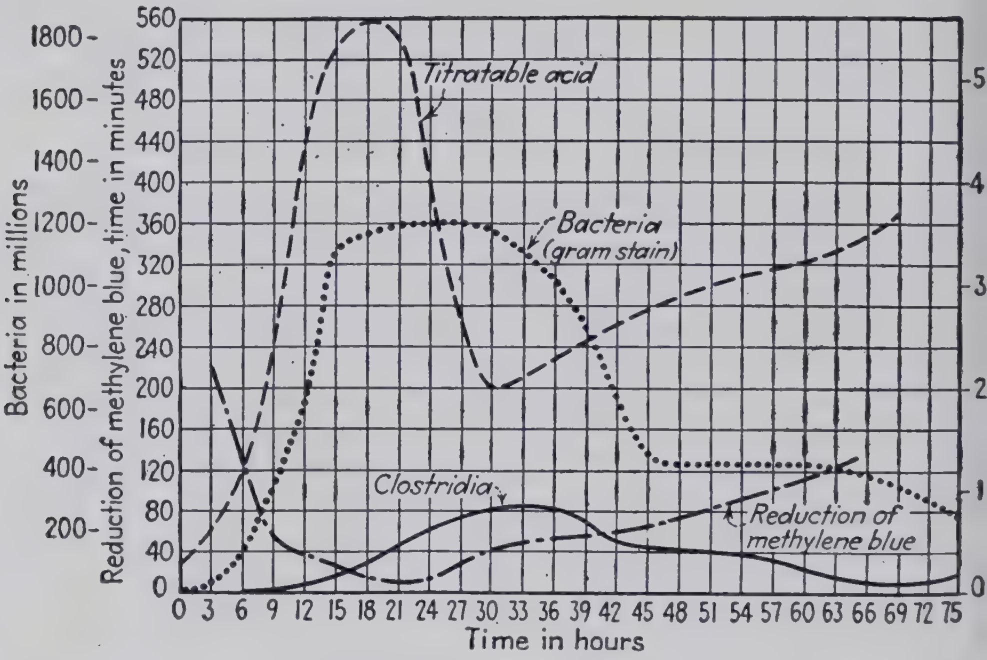
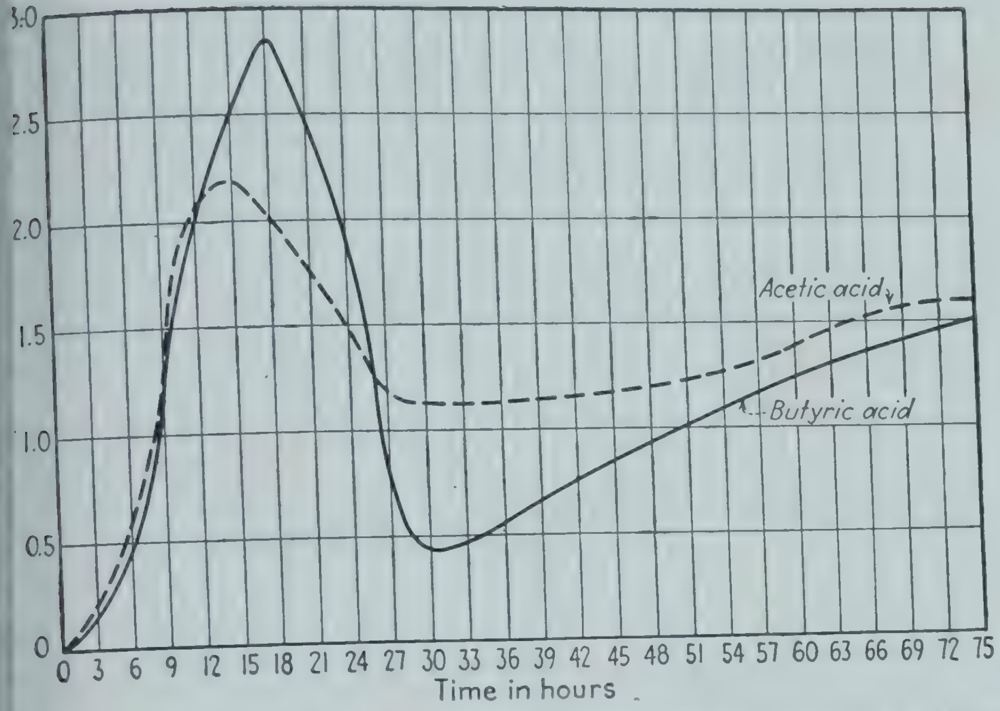
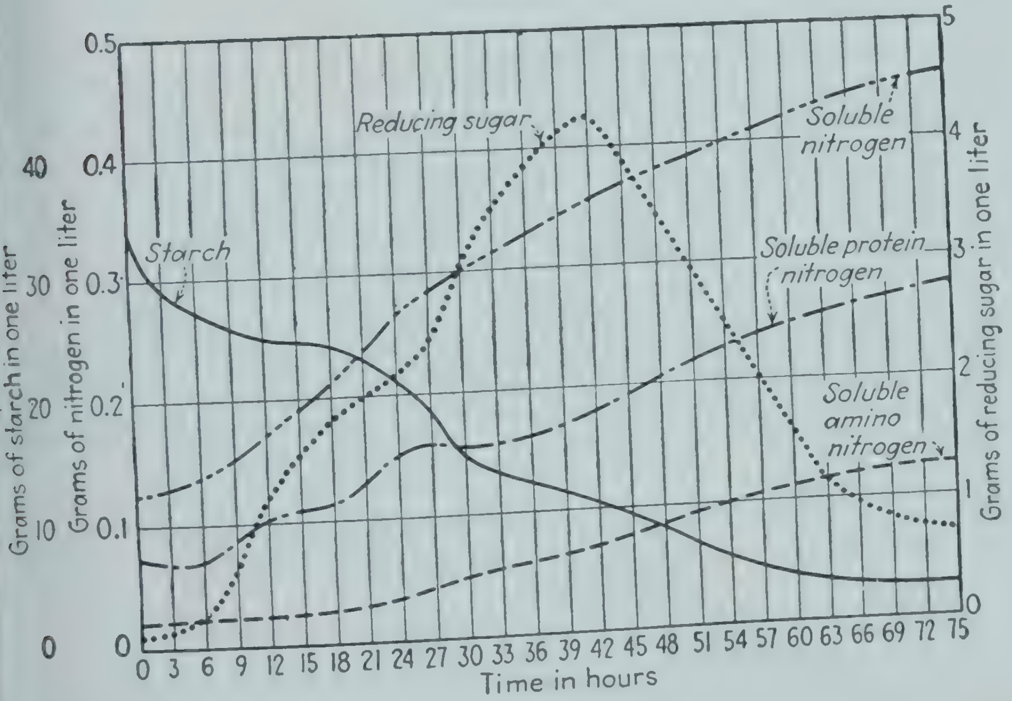


FIG. 42. Numbers of bacteria, time of reduction of methylene blue, and acid p tion at different stages of fermentation. [*Courtesy of W. H. Peterson and E. B. Ind. Eng. Chem.*, 24: 237 (1932). *Copyright* 1932 *by the American Chemical and reprinted by permission of the copyright owner.*]



13. Nature of volatile acids formed by *Clostridium acetobutylicum.* [*Courtesy . H. Peterson and E. B. Fred, Ind. Eng. Chem.,* **24**: 237 (1932). *Copyright* 1932 *ie American Chemical Society and reprinted by permission of the copyright r.*]



44. Changes in carbohydrates and forms of nitrogen caused by *Clostridium obutylicum.* [*Courtesy of W. H. Peterson and E. B. Fred, Ind. Eng. Chem.,* 237 (1932). *Copyright* 1932 *by the American Chemical Society and reprinted by mission of the copyright owner.*]

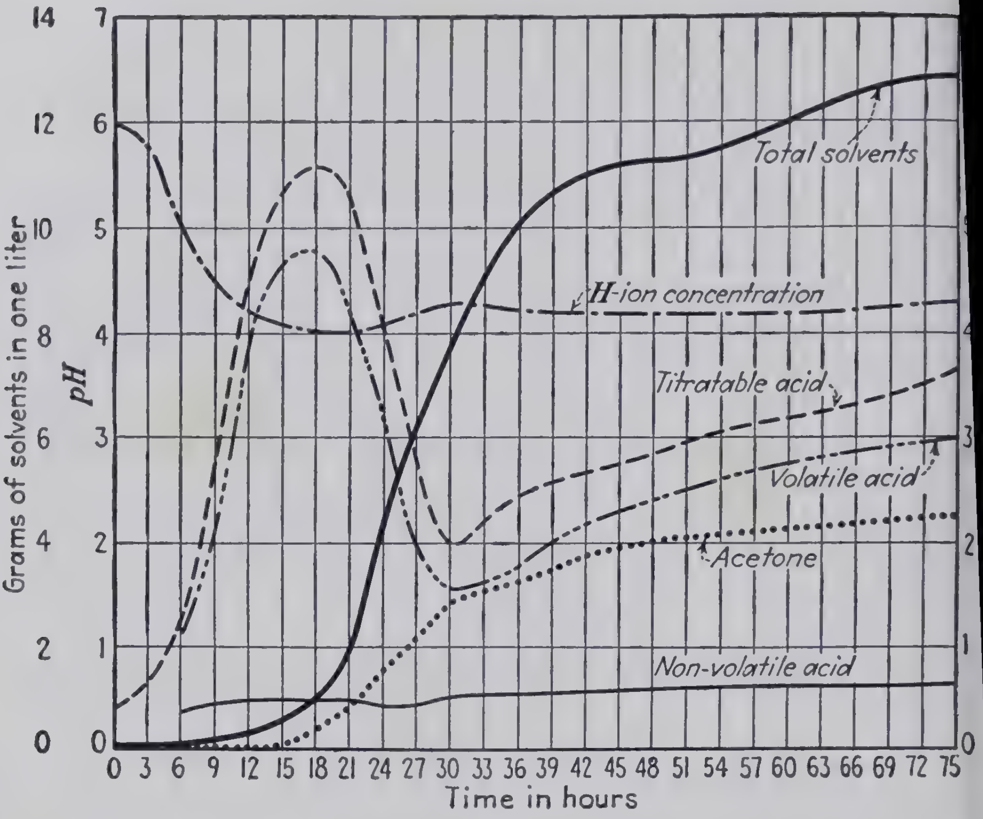


Fig. 45. Acid production and solvent formation. [*Courtesy of W. H. Peterso E. B. Fred, Ind. Eng. Chem.,* 24: 237 (1932). *Copyright* 1932 *by the American ical Society and reprinted by permission of the copyright owner.*]

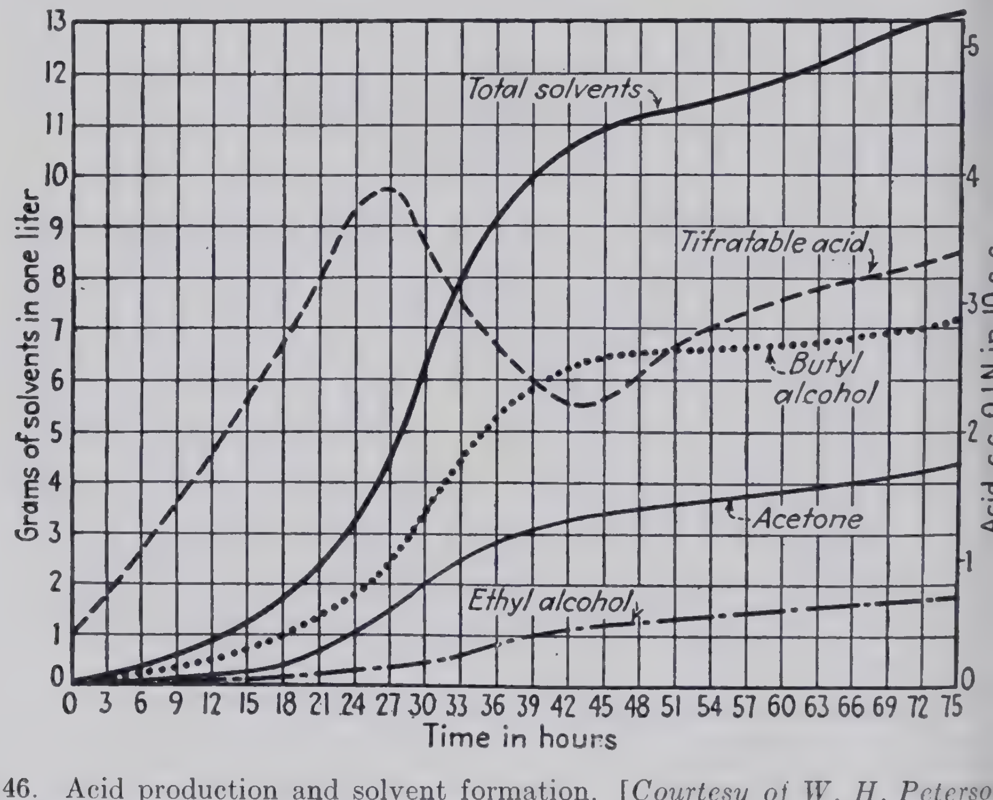
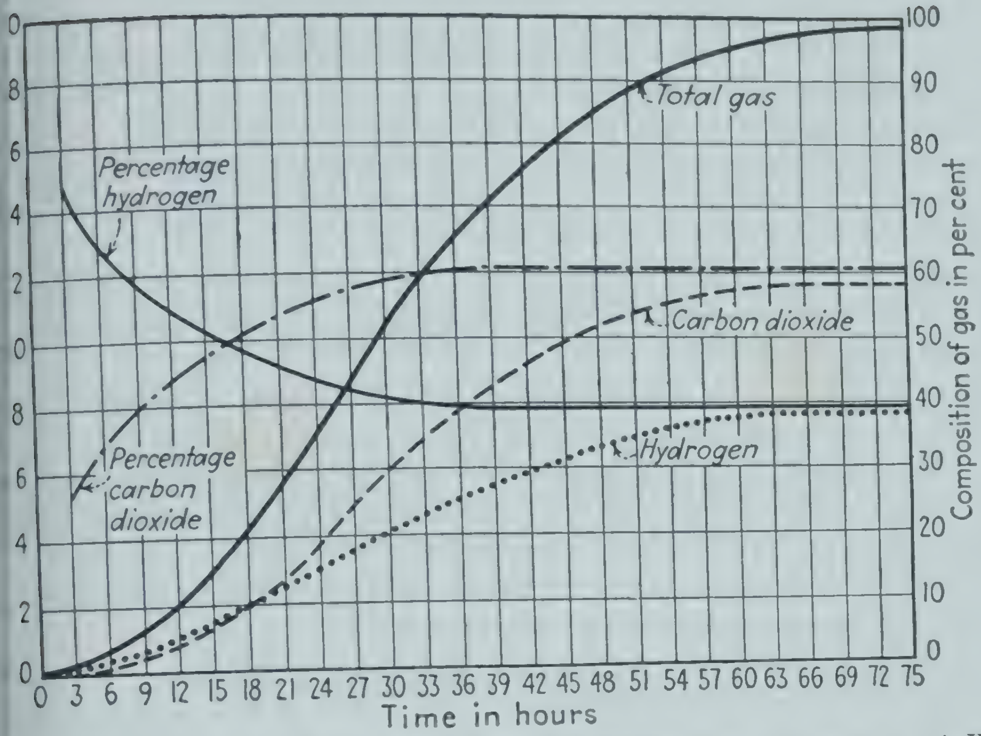


Fig. 46. Acid production and solvent formation. [*Courtesy of W. H. Petersoi E. B. Fred, Ind. Eng. Chem.,* 24: 237 (1932). *Copyright* 1932 *by the Am Chemical Society and reprinted by permission of the copyright owner.*]



17. Production of gas in butyl alcohol fermentation. [*Courtesy of W. H. son and E. B. Fred, Ind. Eng. Chem.*, 24: 237 (1932). *Copyright* 1932 *by the ican Chemical Society and reprinted by permission of the copyright owner.*]

lton, Peterson, and Fred (1926) state that 15 to 60 per cent of the nitrogen of native proteins is rendered soluble during the fermenta- The soluble derivatives of protein (proteoses, peptones, peptides, amino acids) and nonamino nitrogen constitute the soluble nitrogen. de nitrogen is usually the largest single source of soluble nitrogen, ituting 9 to 28 per cent of the total. Amino nitrogen is generally nt in quantities of 7 to 18 per cent, depending upon the type of in being fermented, animal proteins yielding rather high quantities nino nitrogen.

e products formed by the hydrolysis of proteins, a process that ars to progress well at a pH of about 5.5, regulate the pH of the um through buffer action. Butyric and acetic acids are but slightly ciated. Thus high titrable acidity produces very little change in H of the medium.

ctors that cause an increase in acetone frequently produce a decrease ne quantity of ethanol formed. Proteolysis, acid production, and the ntity of solvents produced are affected by the carbohydrate/protein o of the mash. Low carbohydrate/protein ratios favor increased ls of acetone and decreased yields of ethanol. Opposite effects result high carbohydrate/protein ratios. In general, high yields of solvents be obtained with carbohydrate/protein ratios of 5 to 10 (Fulton, rson, and Fred, 1926). High titrable acidity is generally associated a high yield of acetone.

eterson and Johnson (1933) found that *Cl. acetobutylicum* fermented ed acetoacetic acid most rapidly during the period when solvents were being formed at a maximum rate. As much as 3.2 g. of acetoacet per liter has been fermented during 8 hr. in some fermentations, t being decarboxylated to acetone.

Formic acid added to a fermenting mash was destroyed by *Cl. butylicum* (Stiles et al., 1929). This fact strengthens the hypothes formic acid is the precursor of hydrogen and some of the carbon ide gas.

For further discussion of the biochemistry of the butanol ferment the reader is referred to the section dealing with the mechanism fermentation.

**Mechanism for the Formation of End Products.** Before attem to formulate a scheme to show the origin of the products of a ferm tion, it is necessary to assemble some of the known facts. A careful of the charts concerned with the biochemistry of the butanol ferm tion will be most fruitful in this connection.

During the butanol-acetone fermentation, butyric acid disappears rapidly than acetic acid, a fact in accord with the production of a gr proportion of butanol than acetone (Reilly, Hickinbottom, Henley, Thaysen, 1920). The decrease in butyric acid is coincidental with increase in butanol. Additions of butyric acid to a fermenting increase the yield of butanol (Speakman and others). Butyric ac reduced to butanol by cell suspensions when in the presence of glu but not by pyruvate. It is thus probable that glucose, or possibly tr phosphate, is the reducing agent for butyric acid, according to Da (1942). Butyric acid is nearly always found in fermentations w butanol occurs.

Acetic acid disappears from the fermentation medium more slowly t butyric acid, a fact agreeing with the formation of a smaller amoun acetone than butanol. The addition of acetic acid to a fermenting m increases the acetone content but, according to Speakman, does not c any change in the yield of butanol. Acetic acid, acetoacetic acid, pyruvic acid increase the quantity of acetone produced when adde the fermentation (Johnson, Peterson, and Fred, 1933). Propionic ac reduced to propyl alcohol.

Davies (1943) has purified and described the properties of the ac acetic acid decarboxylase of *Cl. acetobutylicum.* The enzyme was spe for acetoacetic acid and had an optimum activity at pH 5.0.

Pyruvic acid is fermented to acetic acid, acetone, and acetylmet carbinol when added to the fermenting mash, but methylglyoxal and a are toxic even in small amounts.

Calcium carbonate in the fermentation mash results in a suppres of butanol and acetone production but causes an increase in the quant of butyric and acetic acids.

A workable scheme must show the derivation of all the end prod in relatively correct proportions. It must be in accord with establis facts of fermentation chemistry. Study of the following schemes show how closely these principles are approached.

Several schemes have been suggested to explain the origin of the

·ts of a normal butanol-acetone fermentation. Fitz suggested the :ion of 4-carbon compounds from 2-carbon compounds by a process densation.

ıkman (1923) was one of the first to suggest a detailed scheme for echanism of the butanol-acetone fermentation. According to him, c and acetic acids were formed by cleavages and oxidations of the molecule. These acids were then reduced in part to the correspond- lvents.

ıberg and Arinstein (Stephenson, 1930) suggested that butyric acid ıtanol were formed from glucose and glycerol through the intermedi- ages of pyruvic acid and pyruvic aldol.

ruvic acid is produced by fermentation from glucose, glycerol, or acid by various microorganisms. But Neuberg and Arinstein were e to increase the yields of butyric acid or butanol through the addi- ɔf a pyruvate to a fermenting medium. They did, however, secure ıses of acetic and formic acids in accordance with the following ion:

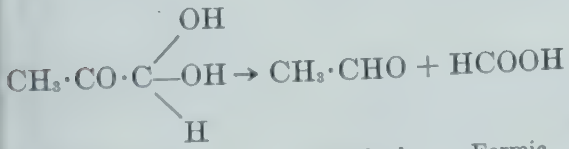
$$\underset{\text{Pyruvic acid}}{CH_3 \cdot CO \cdot COOH} + H_2O \rightarrow \underset{\text{Acetic acid}}{CH_3 \cdot COOH} + \underset{\text{Formic acid}}{HCOOH}$$

uberg and Arinstein therefore concluded that butyric acid and ıol did not arise as a result of the condensation of acetaldehyde and ɔn dioxide. The addition of pyruvic aldol resulted in increased yields ıtyric acid. On the basis of these facts, they formulated their nes.

rious objections have been raised in connection with the schemes ested by Neuberg and Arinstein. The organism that they used, *lus butylicus,* under optimum conditions produced very little butanol. fact that added pyruvic aldol was fermented to butyric acid does not that it was an intermediate product. Even though acetaldehyde not produced from pyruvic acid, this fact cannot be taken as proof it does not arise from some other source.

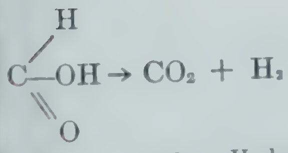
uyver (1931) and his associates have formulated a scheme for the ric acid fermentation, which follows:

$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3$$



$$CH_3 \cdot CO \cdot C(OH)_2H \rightarrow CH_3 \cdot CHO + HCOOH$$

Methylglyoxal hydrate Acetaldehyde Formic acid



$$HC(=O)OH \rightarrow CO_2 + H_2$$

Formic acid Carbon dioxide Hydrogen

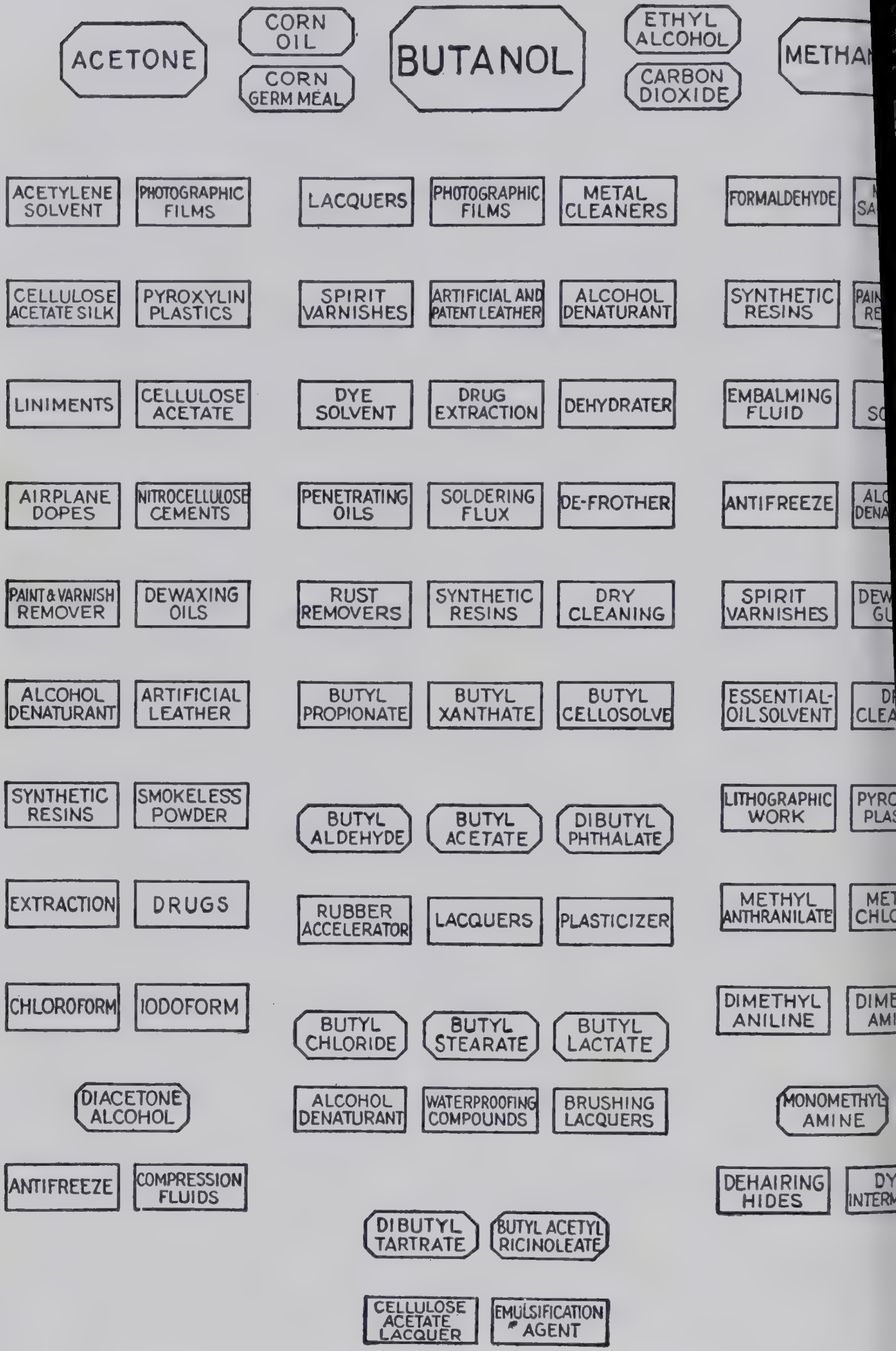
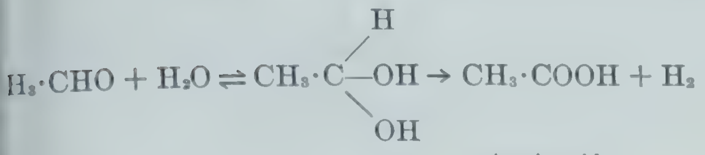
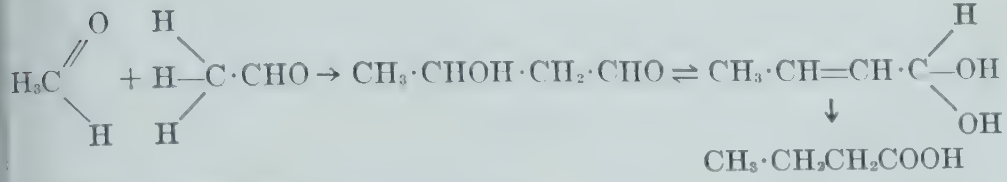

ACETONE
CORN OIL
CORN GERM MEAL
BUTANOL
ETHYL ALCOHOL
CARBON DIOXIDE
METHAN
ACETYLENE SOLVENT
PHOTOGRAPHIC FILMS
CELLULOSE ACETATE SILK
PYROXYLIN PLASTICS
LINIMENTS
CELLULOSE ACETATE
AIRPLANE DOPES
NITROCELLULOSE CEMENTS
PAINT & VARNISH REMOVER
DEWAXING OILS
ALCOHOL DENATURANT
ARTIFICIAL LEATHER
SYNTHETIC RESINS
SMOKELESS POWDER
EXTRACTION
DRUGS
CHLOROFORM
IODOFORM
DIACETONE ALCOHOL
ANTIFREEZE
COMPRESSION FLUIDS
LACQUERS
PHOTOGRAPHIC FILMS
METAL CLEANERS
SPIRIT VARNISHES
ARTIFICIAL AND PATENT LEATHER
ALCOHOL DENATURANT
DYE SOLVENT
DRUG EXTRACTION
DEHYDRATER
PENETRATING OILS
SOLDERING FLUX
DE-FROTHER
RUST REMOVERS
SYNTHETIC RESINS
DRY CLEANING
BUTYL PROPIONATE
BUTYL XANTHATE
BUTYL CELLOSOLVE
BUTYL ALDEHYDE
BUTYL ACETATE
DIBUTYL PHTHALATE
RUBBER ACCELERATOR
LACQUERS
PLASTICIZER
BUTYL CHLORIDE
BUTYL STEARATE
BUTYL LACTATE
ALCOHOL DENATURANT
WATERPROOFING COMPOUNDS
BRUSHING LACQUERS
DIBUTYL TARTRATE
BUTYL ACETYL RICINOLEATE
CELLULOSE ACETATE LACQUER
EMULSIFICATION AGENT
FORMALDEHYDE
SYNTHETIC RESINS
EMBALMING FLUID
ANTIFREEZE
SPIRIT VARNISHES
ESSENTIAL-OIL SOLVENT
LITHOGRAPHIC WORK
METHYL ANTHRANILATE
DIMETHYL ANILINE
MONOMETHYL AMINE
DEHAIRING HIDES

FIG. 48. Chemicals from corn and their uses. (*Courtesy of the Commercial Sol Corp.*)



$$\mathrm{H_3\cdot CHO + H_2O \rightleftharpoons CH_3\cdot \overset{H}{\underset{OH}{C}}{-}OH \rightarrow CH_3\cdot COOH + H_2}$$

Acetic acid



$$\mathrm{\overset{O}{\underset{H}{H_3C}} + H{-}\overset{H}{\underset{H}{C}}\cdot CHO \rightarrow CH_3\cdot CHOH\cdot CH_2\cdot CHO \rightleftharpoons CH_3\cdot CH{=}CH\cdot \overset{H}{\underset{OH}{C}}{-}OH \downarrow CH_3\cdot CH_2CH_2COOH}$$

ətaldehyde Acetaldehyde Acetaldol Butyric acid

the butyric acid fermentation, the main end products are butyric acetic acid, carbon dioxide, hydrogen, and traces of formic acid. uyver is of the opinion that the mechanism for the conversion of · to hydrated methylglyoxal in the butyric acid fermentation is ogous to that of the alcoholic fermentation.

cording to the scheme shown above, the weight of the carbon dioxide ed should be equivalent to approximately one-half of the weight of glucose fermented. (Compare with the ethanol fermentation.) The ber of molecules of acetaldehyde required for the formation of acetic and butyric acid should be equivalent to the number of molecules rbon dioxide produced. Also the total number of molecules of hydrogas evolved (Eqs. 3 and 4 above) should be greater than the number olecules of acetic acid produced (Eq. 4) by the number of molecules arbon dioxide evolved. Table 83 indicates that these requirements reasonably satisfied.

TABLE 83. FERMENTATION BALANCE OF GLUCOSE, USING *Clostridium saccharobutylicum* *

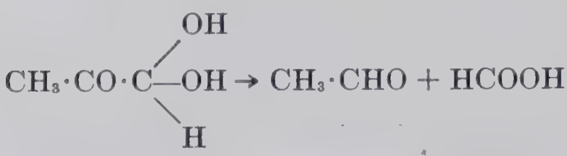
lium: yeast extract, 2 per cent glucose, 1 per cent calcium carbonate, 30°C.)

| Products | Grams | Per cent of glucose fermented | No. of moles/50 moles of glucose fermented | | |
|---|---|---|---|---|---|
| | | | $CO_2$ | $H_2$ | Acetaldehyde |
| :ose added | 39.45 | | | | |
| ·ose unfermented | 3.10 | | | | |
| ·ose fermented | 36.35 | | | | |
| bon dioxide | 17.4 | 47.8 | 97.8 | | |
| lrogen | 0.94 | 2.59 | .... | 116.6 | |
| mic acid | Traces | | | | |
| tic acid | 5.17 | 14.2 | .... | −21.3 | 21.3 |
| yric acid | 13.4 | 36.9 | .... | .... | 75.5 |
| otal | .... | .... | 97.8 | 95.3 | 96.8 |

.A. J. Kluyver, "The Chemical Activities of Micro-organisms," University of ıdon Press, Ltd., London, 1931.

### SCHEME FOR THE BUTYL ALCOHOL FERMENTATION[1]

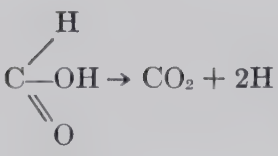
1. $C_6H_{12}O_6 \rightarrow 2C_3H_6O_3$ (by an internal mechanism corresponding to that of the alcoholic fermentation)

2. 

$$CH_3\cdot CO\cdot \overset{OH}{\underset{H}{C}}-OH \rightarrow CH_3\cdot CHO + HCOOH$$

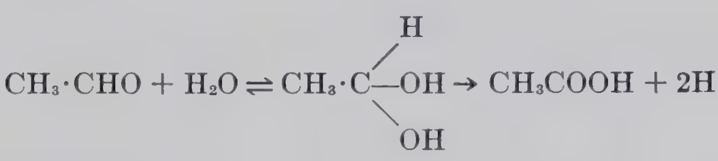
Methylglyoxal hydrate — Acetaldehyde — Formic acid

*Dehydrogenation Reactions*

3. 

$$\overset{H}{\underset{O}{C}}-OH \rightarrow CO_2 + 2H$$

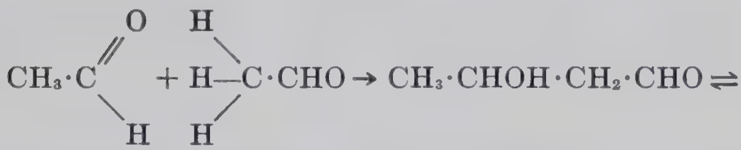
Formic acid

4. 

$$CH_3\cdot CHO + H_2O \rightleftharpoons CH_3\cdot \overset{H}{\underset{OH}{C}}-OH \rightarrow CH_3COOH + 2H$$

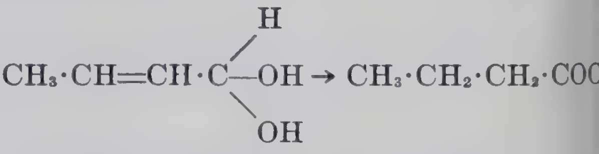
Acetaldehyde — Acetic acid

*Condensation Reactions*

5. 

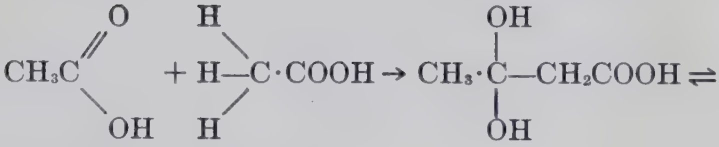
$$CH_3\cdot \overset{O}{\underset{H}{C}} + H-\overset{H}{\underset{H}{C}}\cdot CHO \rightarrow CH_3\cdot CHOH\cdot CH_2\cdot CHO \rightleftharpoons$$

Acetaldehyde — Acetaldehyde — Acetaldol



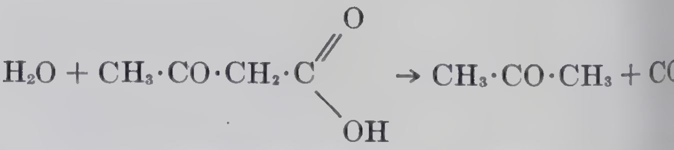
$$CH_3\cdot CH{=}CH\cdot \overset{H}{\underset{OH}{C}}-OH \rightarrow CH_3\cdot CH_2\cdot CH_2\cdot CO$$

Crotonaldehyde hydrate — Butyric acid

6. 

$$CH_3\overset{O}{\underset{OH}{C}} + H-\overset{H}{\underset{H}{C}}\cdot COOH \rightarrow CH_3\cdot \overset{OH}{\underset{OH}{C}}-CH_2COOH \rightleftharpoons$$

Acetic acid — Acetic acid



$$H_2O + CH_3\cdot CO\cdot CH_2\cdot \overset{O}{\underset{OH}{C}} \rightarrow CH_3\cdot CO\cdot CH_3 + C$$

Acetyl acetic acid (Acetoacetic acid) — Acetone — Carl dio

---

[1] A. J. Kluyver, "The Chemical Activities of Micro-organisms," University of L don Press, Ltd., London, 1931. J. B. Van der Lek, "Onderzoekingen over Butylalkoholgisting," Naamlooze Vennootschap W. D. Meinema, Delft, 1930.

SCHEME FOR THE BUTYL ALCOHOL FERMENTATION (*continued*)

*Hydrogenation Reactions*

$H \rightarrow H_2$

$H_3CHO + 2H \rightarrow CH_3 \cdot CH_2OH$
etaldehyde Ethyl alcohol

$H_3 \cdot CH_2 \cdot CH_2 \cdot COOH + 4H \rightarrow CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2OH + H_2O$
Butyric acid Butyl alcohol

$H_3 \cdot CO \cdot CH_3 + 2H \rightarrow CH_3 \cdot CHOH \cdot CH_3$
Acetone Isopropyl alcohol

s necessary to account for several additional products when one from the butyric acid to the butanol-acetone fermentation. A e for this fermentation, to be acceptable, must satisfy conditions pect to the relative proportions of each of the nine or more products in the breakdown of a sugar. The scheme suggested by Kluyver his well, according to the results of Tables 84 and 85, one of which data for a medium containing 2 per cent calcium carbonate.

84. FERMENTATION BALANCE OF GLUCOSE, USING *Clostridium acetobutylicum* *

(Medium: yeast extract, 2 per cent glucose, 37°C.)

| Products | Grams | Per cent of glucose fermented | No. moles/50 moles of glucose fermented | | |
|---|---|---|---|---|---|
| | | | $CO_2$ | $H_2$ | Acetal-dehyde |
| se added .......... | 11.78 | | | | |
| se unfermented .... | 0.41 | | | | |
| se fermented ...... | 11.37 | | | | |
| n dioxide ......... | 6.14 | 54.0 | 110.3 | | |
| gen ............... | 0.1770 | 1.5 | ..... | 70.2 | |
| acid ............ | Traces | | | | |
| acid ............. | 0.53 | 4.7 | ..... | −7.0 | 7.0 |
| acid ............ | 0.24 | 2.1 | ..... | ..... | 4.2 |
| alcohol ........... | 0.27 | 2.4 | ..... | 4.7 | 4.7 |
| alcohol ........... | 2.62 | 23.0 | ..... | 56.0 | 56.0 |
| ne ................ | 0.82 | 7.2 | −11.2 | −22.4 | 22.4 |
| lmethylcarbinol ... | 0.35 | 3.1 | ..... | ..... | 6.4 |
| al ................ | ...... | .... | 99.1 | 101.5 | 100.7 |

B. Van der Lek, "Onderzoekingen over de Butylalkoholgisting," Naamlooze ootschap W. D. Meinema, Delft, 1930.

**udies with Heavy Carbon Acetic and Butyric Acids and Acetone.** d, Brown, and Werkman (1945) studied the mechanism of the butaermentation by *Cl. acetobutylicum* and *Cl. butylicum*, using heavy on acetic and butyric acids and acetone. When heavy carbon acetic $(CH_3 \cdot C^{13}OOH)$ was added to corn-mash fermentations, the heavy

Table 85. Fermentation Balance of Glucose, Using *Clostridium acetobut*

(Medium: yeast extract, 2 per cent glucose, 2 per cent calcium carbonate

| Products | Grams | Per cent of glucose fermented | No. of moles/50 mo glucose ferment |  |
|---|---|---|---|---|
|  |  |  | $CO_2$ | $H_2$ |
| Glucose added .......... | 11.68 |  |  |  |
| Glucose unfermented .... | 0.43 |  |  |  |
| Glucose fermented ...... | 11.25 |  |  |  |
| Carbon dioxide ......... | 5.34 | 47.5 | 97.0 |  |
| Hydrogen .............. | 0.2344 | 2.08 | .... | 93.8 |
| Formic acid ............ | Traces |  |  |  |
| Acetic acid ............ | 1.15 | 10.2 | .... | −15.4 |
| Butyric acid ............ | 2.97 | 26.6 | .... | ..... |
| Ethyl alcohol ........... | 0.39 | 3.5 | .... | 6.8 |
| Butyl alcohol ........... | 0.48 | 4.3 | .... | 10.2 |
| Acetone ................ | Traces |  |  |  |
| Acetylmethylcarbinol ... | 0.28 | 2.5 | .... | ..... |
| Total ................. | ...... | ...... | 97.0 | 95.4 |

* J. B. Van der Lek, "Onderzoekingen over de Butylalkoholgisting," Naan Vennootschap W. D. Meinema, Delft, 1930.

carbon ($C^{13}$) was recovered in butanol, acetone, isopropyl alcohol, bu and acetic acids, ethanol, and carbon dioxide.

The butanol contained about 50 per cent of it, which was prese about equal amounts in the carbinol and beta-positions. Wood an associates suggested that butanol was synthesized from acetic acid derivative of it.

The acetone and isopropyl alcohol contained from 15 to 19 per ce the added heavy carbon atom, which was present in the carbonyl carbinol positions, respectively. The heavy carbon recovery was al the same in respect to these solvents. These facts were in accord the suggestion that acetone is formed by the decarboxylation of a acetic acid, which is produced from acetate.

When heavy carbon butyric acid ($CH_3 \cdot C^{13}H_2CH_2 \cdot C^{13}OOH$) added to the medium, the heavy carbon ($C^{13}$) was recovered in but ethanol, isopropyl alcohol, and acetic and butyric acids. About 8 cent of the added heavy carbon was found in the carbinol and positions of the butanol, suggesting that butyric acid was the prec of this alcohol. It was suggested by Wood and his coworkers that the version of butyric acid to acetic acid, acetone, and isopropyl alcohol represent "a reversible series of reactions through acetoacetic acid."

When heavy carbon acetone ($CH_3 \cdot C^{13}OCH_3$) was added to the r the $C^{13}$ was recovered in the isopropyl alcohol. There was evidence acetone was an intermediate in the formation of isopropyl alcohol.

litional information concerning various aspects of this fermentation
be obtained by reference to the discussions of Barker (1957);
n (1957); Thimann (1955); Gunsalis, Horecker, and Wood (1955);
thers.

### References concerning the Acetone-Butanol Fermentation

ger, C. F., W. H. Peterson, and E. B. Fred: Certain Factors That Influence
etone Production by *Bacillus acetoethylicum, Jour. Biol. Chem.,* **44**: 465–479
920).
yi, S.: Versuche zur Theorie der Aceton-Ethylalkoholischer Gärung, *Biochem.
it.,* **169**: 125 (1926).
n, J. R.: Fermentative Utilization of Cassava, *Iowa State Coll. Jour. Sci.,* **16**:
–18 (1941).
, H. A.: "Bacterial Fermentations," John Wiley & Sons, Inc., New York, 1957.
, S. C.: Acetone-Butanol Fermentation of Sugars, *Ind. Eng. Chem.,* **44**: 1677–
82 (1952).
: Acetone-Butanol Fermentation of Starches, *Appl. Microbiol.,* **1**(2): 85–95
953).
s, J.: Untersuchungen über die Gewinnung der Hanfasser durch naturliche
stmethoden, *Zentr. Bakt. Parasitenk.,* Abt. II, **8**: 114 (1902).
nck, M. W.: Über die Butylalkoholgärung und das Butyl-ferment, *Ver-
ndel. Akad. Wetenschappen Amsterdam, Afdeel. Natuurkunde,* Sectie II, Deel I,
93.
: Sur la fermentation et le ferment butyliques, *Arch. néerland.,* **29**: 1–68 (1896).
: Sur les bacteries actives dans le Rouissage du Lin, *Arch. néerl. Sci.,* Ser. II,
436–441 (1904).
y's Manual of Determinative Bacteriology," 7th ed., The Williams & Wilkins
mpany, Baltimore, 1957.
uer, K., und K. Kurschner: Butyl- und Aceton- Gärungen. I. Mitteilung:
er Zwischenprodukte der Butanol-Aceton-Gärung, *Biochem. Zeit.,* **280**: 379–
7 (1935).
lot, A., et E. Ossart: Recherches sur les microbes producteurs d'acetone,
*mpt. rend.,* **173**: 792–794 (1921).
, S.: Über einen Bacillus Butyricus, *Zeit. Hyg. Infektionskrankh.,* **11**: 421–434
392).
ann, G.: *Bacillus amylobacter* A. M. et Bredemann, *Zentr. Bakt. Parasitenk.,*
t. II, **23**: 385–568 (1909).
, R. W., H. G. Wood, and C. H. Werkman: Growth Factors for the Butyl
cohol Bacteria, *Jour. Bact.,* **25**: 206 (1938).
, L. C., L. M. Christensen, and E. I. Fulmer: Hydrolysis of Oat Hulls with
drochloric Acid, *Ind. Eng. Chem.,* **28**: 206–208 (1936).
r, E.: Hoppe-Seyler. Über den Einfluss des Sauerstoffs auf Gärungen, *Zeit.
ysiol. Chem.,* **9**: 380–415 (1885).
und J. Meisenheimer: Über Buttersäuregärung, *Ber.,* **41**: 1410–1419 (1908).
ensen, L. M., and E. I. Fulmer: Analysis of *n*-Butanol, Acetone, and Ethanol
Aqueous Solution, *Ind. Eng. Chem. (Anal. Ed.),* **7**: 180–182 (1935).
ensen, W. B., M. J. Johnson, and W. H. Peterson: Properties of the Lactic
id-racemizing Enzyme of *Clostridium butylicum, Jour. Biol. Chem.,* **127**: 431
939).
, C. E.: "Introduction to Bacterial Physiology," McGraw-Hill Book Company,
c., New York, 1957.
, G. N.: Nature et mode de formation des acides volatils trouvés dans les cul-
res de bactéries anaérobies strictes, *Ann. inst. Pasteur,* **77**: 471–511 (1949).
: Metabolism of Bacteria, *Ann. Rev. Microbiol.,* **5**: 71–100 (1951).
, R.: Studies in the Acetone-Butyl Alcohol Fermentation. 2. Intermediates in
e Fermentation of Glucose by *Cl. acetobutylicum,* 3. Potassium as an Essential

Factor in the Fermentation of Maize Meal by *Cl. acetobutylicum* (BY) *chem. Jour.*, **36**: 582–599 (1942).

———: Studies on the Acetone-Butanol Fermentation. 4. Acetoacetic Acid D boxylase of *Cl. acetobutylicum* (BY), *Biochem. Jour.*, **37**: 230–238 (1943).

——— and M. Stephenson: Studies on the Acetone-Butyl Alcohol Ferment I. Nutritional and Other Factors Involved in the Preparation of Active Su sions of *Cl. acetobutylicum* (Weizmann), *Biochem. Jour.*, 1320–1331 (

Duclaux, E.: Sur la nutrition intracellulaire, *Ann. inst. Pasteur*, **9**: 811–839 (

Dunn, C. G.: "A Biochemical Investigation of the Metabolic Activities of *Ba Technicus*," Ph.D. thesis, Massachusetts Institute of Technology, Departme Biology and Public Health, Cambridge, Mass., 1934.

Dunning, J. W., and E. C. Lathrop: The Saccharification of Agricultural Resi *Ind. Eng. Chem.*, **37**(1): 24–29 (1945).

Emmerling, O.: Butylalkoholische Gärung, *Ber.*, **30**: 451–453 (1897).

Fitz, A.: *Bacillus butylicus*, *Ber.*, **15**: 867–880 (1882).

———: Über Spaltpilzgärungen, *Ber.*, **17**: 1188 (1884).

Fowler, G. J., and V. Subramanyan: Studies Relating to the Acetone Producin ganisms, *Jour. Indian Inst. Sci.*, **8A**: 71–87 (1925).

———, Y. D. Wad, and A. G. Gokhale: Acetone Fermentation Process in I *Jour. Indian Inst. Sci.*, **4**: 1–15 (1921).

Fred, E. B., W. H. Peterson, and J. A. Anderson: Production of Acetone, Alc and Acids from Oat and Peanut Hulls, *Ind. Eng. Chem.*, **15**: 126 (1923).

———, ———, and W. R. Carrol: The Occurrence of a Red Pigment Produ Organism in Corn Mash of the Acetone Butyl Alcohol Fermentation, *Jour. B* **10**: 97–104 (1925).

———, ———, and M. Mulvania: The Effect of Lactic Acid Bacteria on the Acet Butyl Alcohol Fermentation, *Jour. Bact.*, **11**: 323–343 (1926).

Freiberg, G. W.: Observation on the Carbohydrate Metabolism of Acetone Bu Alcohol Fermentations, *Proc. Soc. Exptl. Biol. Med.*, **23**: 72–73 (1925).

Fulton, H. L., W. H. Peterson, and E. B. Fred: The Hydrolysis of Native Pro by *B. granulobacter pectinovorum* and the Influence of the Carbohydrate-pro Ratio on the Products of Fermentation, *Zentr. Bakt. Parasitenk.*, Abt. II, 1–11 (1926).

Gabriel, C. L.: Butanol Fermentation Process, *Ind. Eng. Chem.*, **20**: 1063– (1928).

——— and F. M. Crawford: Development of the Butyl-Acetone Fermentation In try, *Ind. Eng. Chem.*, **22**: 1163–1165 (1930).

Gfeller, P.: Mikrobiologische Verwertung der Sulfitablaugen auf dem Garwege u besonderer Berücksichtigung technisch verwendbarer Produkte, dissertation Eidgenössichen Technischen Hochschule, Zurich, 1954.

Gill, A.: The Acetone Fermentation Process and Its Technical Application, *J Soc. Chem. Ind.*, **38**: 273–282T (1919).

———: The Production of Normal Butyl Alcohol and Acetone by Fermentatio Horse Chestnuts, *Jour. Soc. Chem. Ind.*, **38**: 411–412T (1919).

Grassberger, R., und A. Schattenfroh: Über Buttersäuregärung, *Arch. Hyg.*, **42**: 246 (1902).

Grimbert, M. L.: Fermentation anaérobie produite par le *Bacillus orthobutyl* *Ann. inst. Pasteur*, **7**: 353–402 (1893).

Gruber, M.: Eine Methode der Cultur anaerobischer Bacterien Bemerkungen nebst Morphologie der Buttersäuregärung, *Zentr. Bakt. Infektionskrankh.*, 367–372 (1887).

Gunsalis, I. C., B. L. Horecker, and W. A. Wood: Pathways of Carbohydrate Meta lism in Microorganisms, *Bact. Revs.*, **19**: 79–128 (1955).

Higgins, G. A.: Solvents from Kelp, *Ind. Eng. Chem.*, **10**: 858–859 (1918).

Kelly, F. C.: "One Thing Leads to Another," Houghton Mifflin Company, Bos 1936.

Kentner, J.: Über das Vorkommen und die Verbreitung stickstoffbindender Bakte in Meere, *Wiss. Meeresuntersuch.*, N. F. 8, 1904, p. 42.

fer, D. H.: Butanol and Acetone from Corn, *Ind. Eng. Chem.*, **19**: 46–50
1927).
er, A. J.: "The Chemical Activities of Micro-organisms," University of London
ress, Ltd., London, 1931.
-: Die bakteriellen Zuckervergärungen, *Ergeb. Enzymforsch.*, **4**: 230–273 (1935).
si, G., and S. R. Dutky: The Growth of a Butanol *Clostridium* in Relation to
ie Oxidation-reduction Potential and Oxygen Content of the Medium, *Jour.
act.*, **31**: 137–149 (1936).
ykke, A. F., and E. B. Fred: Products from the Fermentation of Glucose and
rabinose by Butyric Acid Anaerobes, *Jour. Bact.*, **29**: 86–87 (1935).
-, W. H. Peterson, and E. B. Fred: Reductive Processes of *Clostridium butyli-
ım* and the Mechanism of Formation of Isopropyl Alcohol, *Jour. Bact.*, **34**: 443–
53 (1937).
-, ———, and E. McCoy: Products from the Fermentation of Glucose and
rabinose by Butyric Acid Anaerobes, *Jour. Bact.*, **29**: 333–347 (1935).
-, J. M. Van Lanen, and D. R. Fraser: Butyl Alcohol from Xylose Sacchari-
ation Liquors from Corncobs, *Ind. Eng. Chem.*, **40**(9): 1716–1719 (1948).
ırd, R. H., W. H. Peterson, and G. J. Ritter: Butanol-Acetone Fermentation
f Wood Sugar, *Ind. Eng. Chem.*, **39**: 1443–1445 (1947).
ung, L. S., E. McCoy, and E. B. Fred: Further Extensive Uses of the Vegetative
issue Anaerobic System, *Zentr. Bakt. Parasitenk.*, Abt. II, **91**: 225–227 (1935).
y, E., E. B. Fred, W. H. Peterson, and E. G. Hastings: A Cultural Study of
ie Acetone Butyl Alcohol Organism, *Jour. Infectious Diseases*, **39**: 457–483
1926).
-, ———, ———, and ———: A Cultural Study of Certain Anaerobic Butyric-
cid Forming Bacteria, *Jour. Infectious Diseases*, **46**: 118–137 (1930).
- and L. S. McClung: The Serological Agglutination of *Clostridium acetobutyli-
ım* and Related Species, *Jour. Infectious Diseases*, **56**: 333–346 (1935).
ıniel, L. E., D. W. Woolley, and W. H. Peterson: Growth Factors for Bacteria.
II. Nutritional Requirements of Certain Butyl-alcohol Producing Bacteria,
*our. Bact.*, **37**: 259 (1939).
el, C. S., and A. E. Broderick: The Composition of Yellow Oil Obtained in
e Manufacture of *n*-Butyl Alcohol by Fermentation, *Jour. Am. Chem. Soc.*,
: 3045–3051 (1925).
awa, K.: "A New Butyl and Isopropyl Alcohol Fermentation," Ph.D. thesis,
lassachusetts Institute of Technology, Department of Biology and Public
ealth, Cambridge, Mass., 1925.
- and S. C. Prescott: A New Fermentation Yielding Butyl and Isopropyl Alco-
ols, *Jour. Bact.*, **13**: 58 (1928).
n, F.: The Manufacture of Acetone, *Jour. Soc. Chem. Ind.*, **38**: 271–273T
919).
E. F.: Chemical Processes in Fermentations, *Chem. Rev.*, **3**: 41–79 (1926).
rop, J. H., L. H. Ashe, and J. K. Senior: Biochemistry of *Bacillus Acetoethyli-
ım* with Reference to the Formation of Acetone, *Jour. Biol. Chem.*, **39**: 1–21
1919).
n, O. L.: The Production of Butyl and Isopropyl Alcohols by Fermentative
rocesses, *Iowa State College Jour. Sci.*, **10**: 97–98 (1935).
ur, L.: Extrait des procés-verbaux, *Soc. Chim. Paris* (*Bull.*), 1862, p. 52.
ix, M. L.: Sur les fermentations produites par un microbe anaérobie de l'eau,
*Inn. inst. Pasteur*, **5**: 287–311 (1891).
son, W. H., and E. B. Fred: Butyl-acetone Fermentation of Corn Meal, *Ind.
ng. Chem.*, **24**: 237 (1932).
-, ———, and B. P. Domogalla: The Proteolytic Action of *Bacillus granulo-
acter pectinovorum* and Its Effect on the Hydrogen-ion Concentration, *Jour.
m. Chem. Soc.*, **46**: 2086–2090 (1924).
-, ———, and E. G. Schmidt: The Fermentation of Pentoses by *Bacillus
ranulobacter pectinovorum*, *Jour. Biol. Chem.*, **60**: 627–631 (1924).
-, ———, and J. H. Verhulst: A Fermentation Process for the Production of

Acetone, Alcohol, and Volatile Acids from Corncobs, *Ind. Eng. Chem.*, 13: 759 (1921).

——— and M. J. Johnson: Fermentation of Acetoacetic Acid and Pyruvic Aci the Acetone-Butanol Organism, *Clostridium acetobutylicum, Jour. Bact.*, 2 (1933).

Pfeifer, V. F., and C. Vojnovich: Continuous Sterilization of Media in Bioche Processes, *Ind. Eng. Chem.*, **44**: 1940–1946 (1952).

Prazmowski, A.: Zur Entwickelungsgeschichte und Fermentwirkung einiger terien-Arten, *Botan. Zeit.*, **37**: 409–422 (1879).

Prescott, S. C., and K. Morikawa: Production of Butyl and Isopropyl Alco U.S. Patent 1,933,683.

Pringsheim, H.: Über ein Stickstoff assimilierendes Clostridium, *Zentr. Bakt. sitenk.*, Abt. II, **16**: 795–800 (1906).

Reilly, J., and W. J. Hickinbottom: Mechanism of the "*n*-Butyl Alcohol Acetone" Fermentation Process, *Chem. News*, **119**: 169–170 (1919).

———, ———, F. R. Henley, and A. C. Thaysen: The Products of the "Ace *n*-Butyl Alcohol" Fermentation of Carbohydrate Material with Special R ence to Some of the Intermediate Substances Produced, *Biochem. Jour.*, 229–251 (1920).

Reinke, J., und G. Berthold: Die Zersetzung der Kartoffel durch Pilze, *Unters botan. lab. Univ. Gottingen,* 1879, p. 1.

Robinson, G. C.: A Study of the Acetone and Butyl Alcohol Fermentation of Var Carbohydrates, *Jour. Biol. Chem.*, **53**: 125–154 (1922).

Schardinger, F.: *Bacillus macerans*, ein Aceton bildender Rottebacillus, *Zentr. B Parasitenk.*, Abt. II, **14**: 772–781 (1905).

———: Verhalten von Weizen und Roggenmehl zu Methylenblau und zu Sta kleister, *Zentr. Bakt. Parasitenk.*, Abt. II, 748–767 (1907).

Schoen, M.: "The Problem of Fermentation," Chapman & Hall, Ltd., London, 1

Sjolander, N. O.: The Fermentation Products of *Clostridium thermosaccharolytic Jour. Bact.*, **34**: 419–428 (1937).

——— and E. McCoy: A Cultural Study of Some "Butyric" Anaerobes Previo Described in the Literature, *Zentr. Bakt. Parasitenk.*, Abt. II, **97**: 314–324 (19

———, A. F. Langlykke, and W. H. Peterson: Butyl Alcohol Fermentation of W Sugar, *Ind. Eng. Chem.*, **30**: 1251 (1938).

Smyth, H. F., and W. L. Obold: "Industrial Microbiology," The Williams & Will Company, Baltimore, 1930.

Speakman, H. B.: The Production of Acetone and Butyl Alcohol by a Bacteriolog Process, *Jour. Soc. Chem. Ind.*, **38**: 155–161T (1919).

———: Seed-culture Methods in the Production of Acetone and Butyl Alcohol b Fermentation Process, *Ind. Eng. Chem.*, **12**: 581–587 (1920).

———: The Biochemistry of the Acetone and Butyl Alcohol Fermentation of Sta by *Bacillus granulobacter pectinovorum, Jour. Biol. Chem.*, **41**: 319–343 (1920

———: Gas Production during the Acetone and Butyl Alcohol Fermentation Starch, *Jour. Biol. Chem.*, **43**: 401–411 (1920).

———: Molecular Configuration in the Sugars and Acid Production by *Baci granulobacter pectinovorum, Jour. Biol. Chem.*, **58**: 395–413 (1923).

———: The Biochemistry of Acetone Formation from Sugars by *Bacillus acetoeth cum, Jour. Biol. Chem.*, **64**: 41–52 (1925).

———: The Physiological Significance of Deamination in Relation to Glucose Oxi tion, *Jour. Biol. Chem.*, **70**: 135–150 (1926).

——— and J. F. Phillips: A Study of a Bacterial Association, *Jour. Bact.*, **9**: 183– (1924).

Stadtman, E. R., and H. A. Barker: Fatty Acid Synthesis by Enzyme Preparati of *Clostridium kluyveri, Jour. Biol. Chem.*, **180**: 1085–1093, 1095–1115, 1117–11 1169–1186 (1949); **181**: 221–235 (1949); **184**: 769–793 (1950).

——— and T. C. Stadtman: Metabolism of Microorganisms, *Ann. Rev. Microbi* 7: 143–178 (1953).

Stephenson, Marjory: "Bacterial Metabolism," Longmans, Green & Co., Inc., N York, 1930.

H. R., W. H. Peterson, E. B. Fred: The Nature of the Acids Produced in e Fermentation of Maize by *Clostridium acetobutylicum*, *Jour. Biol. Chem.*, **4**: 437–453 (1929).

er, K., Über die Wasserroste des Flachses, *Zentr. Bakt. Parasitenk.*, Abt. II, **3**: 171 (1904).

n, E. L., W. H. Peterson, and E. B. Fred: An Unknown Factor Stimulating the ormation of Butyl Alcohol by Certain Butyric Acid Bacteria, *Jour. Bact.*, **27**: 07–217 (1934).

–, ———, and ———: Identification of Asparagine as the Substance Stimulat- g the Production of Butyl Alcohol by Certain Bacteria, *Jour. Bact.*, **29**: 563–72 (1935).

sen, A. C.: Bacteriology of the Process for Acetone and Butyl Alcohol Manu- acture, *Jour. Inst. Brewing*, **27**: 529–542 (1921).

ann, K. V.: "The Life of Bacteria," The Macmillan Company, New York, 955.

kofler, L. A., L. M. Christensen, and E. I. Fulmer: Butyl-acetonic Fermenta- on of Xylose and Other Sugars, *Ind. Eng. Chem.*, **28**: 350–354 (1937).

–, E. I. Fulmer, and M. M. Rayman: Oat Hull Utilization by Fermentation, *d. Eng. Chem.*, **29**: 1290–1292 (1937).

– and J. E. Hunter, Jr.: Butyl-acetonic Fermentation of Arabinose and Other ugars, *Ind. Eng. Chem.*, **30**: 480 (1938).

der Lek, J. B.: "Onderzoekingen over de Butylalkoholgisting," thesis, Tech- ische Hoogeschool, Delft, 1930.

Tieghem, M.: Sur le *Bacillus amylobacter* et son rôle dans la putréfaction des issues végétaux, *Bull. soc. botan. France*, **24**: 128–135 (1877).

–: Identité du *Bacillus amylobacter* et du vibrion butyrique de M. Pasteur, *ompt. rend.*, **89**: 5–8 (1879).

Klecki, V.: Ein neuer Buttersäuregärungserreger, *Zentr. Bakt. Parasitenk.*, bt. II, **2**: 169–184 (1896).

man, S., and D. Kirsh: Butyric Acid and Butyl Alcohol Fermentation of Hemi- ellulose- and Starch-rich Materials, *Ind. Eng. Chem.*, **25**: 1036–1041 (1933).

r, H. H., und W. Koch: Zur Methodik der Analyse technischer Lösungsmittel, *hem. Ztg.*, **57**: 73–74 (1933).

stein, L., and L. F. Rettger: Some Factors Involved in the Biological Produc- on of Acetone and Butyl Alcohol, *Jour. Bact.*, **23**: 74–75 (1932).

land, R. T., E. I. Fulmer, and L. A. Underkofler: Butyl-Acetonic Fermenta- on of Jerusalem Artichokes, *Ind. Eng. Chem.*, **33**: 1078–1081 (1941).

r, E. R., and L. F. Rettger: A Comparative Study of Six Different Strains of e Organism Commonly Concerned in Large-scale Production of Butyl Alcohol nd Acetone by the Biological Process, *Jour. Bact.*, **14**: 399–424 (1927).

A. J., M. J. Johnson, E. McCoy, and W. H. Peterson: Acetone-Butyl Alcohol ermentation of Waste Sulfite Liquor, *Ind. Eng. Chem.*, **35**: 606–610 (1941).

n, P. W., W. H. Peterson, and E. B. Fred: The Production of Acetylmethyl arbinol by *Clostridium acetobutylicus*, *Jour. Biol. Chem.*, **74**: 495–507 (1927).

–, ———, and ———: The Relationship between the Nitrogen and Carbon etabolism of *Clostridium acetobutylicum*, *Jour. Bact.*, **19**: 231–260 (1930).

gradsky, S.: *Clostridium pastorianum*, seine Morphologie und seine Eigenschaften ls Buttersäureferment, *Zentr. Bakt. Parasitenk.*, Abt. II, **9**: 43–54, 107–112 (1902).

l, H. G., R. W. Brown, and C. H. Werkman: Mechanism of the Butyl Alcohol ermentation with Heavy Carbon Acetic and Butyric Acids and Acetone, *Arch. iochem.*, **6**: 243–260 (1945).

oop, R.: *n*-Butanol and Acetone, *Ind. Eng. Chem.*, **35**: 1240–1242 (1943).

e, A. M.: Inhibition of the Acetone-butyl Alcohol Fermentation by Acids, *our. Bact.*, **22**: 209–237 (1931).

### Some Patents

rger, C. F.: Process for the Production of Butyl Alcohol by Fermentation, S. Patent 2,050,219, Aug. 4, 1936.

Bayer & Co.: Production of Acetone and Alcohol by Fermentation of Suga Saccharine Materials by *Bacillus Macerans,* German Patent 283107, 1913.

Boinot, F.: Manufacture of Normal Butyl Alcohol, U.S. Patent 1,565,543, De 1925.

Carnarius, E. H.: U.S. Patent 2,423,580, 1947.

Desborough, A. P. H., A. C. Thaysen, J. Reilly, and F. R. Henley: The Manufa of Acetone and Other Products by Fermentation, British Patent 128,714, 191

Edmonds, W. J.: Butyl Acetonic Fermentation Process, U.S. Patent 1,740,162, 17, 1929.

Fernbach, A.: Acetono-butylic Fermentation Process, U.S. Patent 1,854,895, 19, 1932.

——— and E. H. Strange: Acetone and Higher Alcohols (Amyl, Butyl or Alcohols and Butyric, Propionic or Acetic Acid) from Starches, Sugars, and Carbohydrates, British Patents 15,203–15,204, 1911.

——— and ———: Fermentation Process for the Production of Acetone and H Alcohols from Starch, Sugars and Other Carbohydrate Material, U.S. P 1,044,368, Nov. 12, 1912.

——— and ———: Manufacture and Production of a Nutrient Medium for U Fermentation Processes, U.S. Patents 1,044,446–1,044,447, Nov. 12, 1912.

——— and ———: Production of Acetone, Acetates and Pyruvates, British Pa 14,607, 1915.

Freiberg, G. W.: Process for Producing Acetone and Butyl Alcohol, U.S. Pa 1,537,597, May 12, 1925.

———: Fermentation Process for Production of Acetone and Butyl Alcohol, Patent 1,551,550, Sept. 1, 1925.

———: Process for Making Acetone and Butyl Alcohol, U.S. Patent 1,672,487, Ju 1928.

Hancock, C. W.: U.S. Patent 1,969,218, 1934.

Legg, D. A., and H. R. Stiles: Process of Producing Butyl Alcohol, U.S. Pa 2,089,562, Aug. 10, 1937.

McCutchan, W. N., and C. F. Arzberger: Production of Butyl Alcohol by Ferme tion, U.S. Patent 2,023,087, Dec. 3, 1935.

CHAPTER 14

# THE BUTANOL-ISOPROPANOL FERMENTATION

e fermentation in which butanol and isopropanol are formed as the principal products of commercial value is related to the acetone-10l fermentation and the acetone-ethanol fermentation.

ijerinck (1893) isolated an organism that produced butanol from sugar. This microbe, although described as *Granulobacter butyli-* has since been designated as *Clostridium butylicum* (Beijerinck) ker.

e occurrence of isopropanol ($CH_3 \cdot CHOH \cdot CH_3$) as a fermentation ıct was observed by Pringsheim (1906). The organism that gave o isopropanol was named *Cl. americanum* Pringsheim. This organs now regarded as possibly identical with *Cl. butylicum* (Beijerinck) ker.

lpmers (1920) isolated *Granulobacter butylicum* [*Cl. butylicum* erinck) Donker] from malt and demonstrated that the products of entation included butanol and isopropanol; acetic, butyric, and other ; and gases.

ɔrikawa (1926) isolated an organism, designated as *Bacillus techni-* 'rescott and Morikawa, which produced butanol, isopropanol, traces ıall amounts of acetone and acetic and butyric acids, and hydrogen arbon dioxide from *koji* mashes. *B. technicus* appeared to differ in al respects from *Cl. butylicum*. The fermentation brought about by nicrobe has been studied by Morikawa and Prescott (1927) and (1934).

*butylicum* (Beijerinck) Donker was studied by Van der Lek (1930).

**me General Considerations concerning the Fermentation.** There everal organisms that have the ability to produce isopropyl alcohol. e are closely related. In view of the fact that *Cl. butylicum* (Beick) Donker is perhaps the best known of the isopropyl alcohol pro-rs, this organism and the fermentations it produces will be considered me detail.

*scription of* Cl. butylicum.[1] *Cl. butylicum* is a sporeforming oblianaerobe, possessing peritrichous flagella. It is Gram-positive in g cultures but in old cultures may be Gram-negative. The vegetative of a 24-hr. culture grown at 37°C. in a 4 per cent corn mash containing potato extract are rod-shaped, 2 to 5 μ in length and 0.7 to 1.5 width. These cells, which have rounded ends, occur singly, in pai in chains. Granulose is present in young cells. Old cultures co many spores.

---

[1] urtesy of O. L. Osburn, R. W. Brown, and C. H. Werkman, *Iowa State Coll. Sci.*, **12**: 275 (1938).

The optimum temperature for growth is 37°C.

*Cl. butylicum* does not produce indol; reduce nitrates to nitrites; li starch; assimilate peptone, unless carbohydrates are present; or u ammonium salts. It is catalase-negative and forms hydrogen sulfide sulfites, thiosulfites, and oatmeal.

Acid and gas are formed from amygdalin, arabinose, cellobiose, de dimethylglucoside, esculin, galactose, glucose, glycogen, inositol, in lactose, levulose, maltose, melibiose, melezitose, raffinose, rhamnose, cin, starch, sucrose, trehalose, and xylose. No acids or gas are fo from adonitol, dulcitol, erythritol, glycerol, mannitol, pectin, sodium tate, or sorbitol.

The final products include butyl and isopropyl alcohols, carbon di and hydrogen, small quantities of butyric and acetic acids, and pos traces of acetone and formic acid.

*Nitrogen Sources.* Some of the most favorable sources of nitroge the butyl-isopropyl alcohol organisms are yeast extract, peptone, sprouts, and corn-steep liquor. Each of these nitrogenous substrates tains protein in a partially hydrolyzed form. Other partially hydro proteins may be used as sources of nitrogen. *Cl. butylicum* does hydrolyze proteins appreciably.

Malt sprouts have been used by Beijerinck, Folpmers, Osburn Werkman, and others. One sample of malt sprouts used by Osburn Werkman contained 3.52 per cent of nitrogen, 10 per cent of mal and 18 per cent of starch and dextrin. Of the nitrogenous material, 3 cent was soluble in hot water and 30 per cent was in the form of a nitrogen.

Osburn and Werkman demonstrated that 5 to 11 per cent of isopr alcohol and 19.5 to 25 per cent of butanol could be produced from cent glucose media containing mixtures of corn-steep water and sprouts or corn gluten (the proteins of corn gluten are zein and glu principally).

Low yields of butanol (3 to 4 per cent) were obtained in the ferme tion of 4 per cent corn mashes by *Cl. butylicum,* but the addition of of yeast extract, or 5 ml. of corn-steep water, per 300 ml. of mash resu in vigorous fermentations that went to completion with the produc of 12 to 14 per cent of butanol (Osburn and Werkman, 1935).

The addition of asparagine (Brown et al., 1938), yeast extract, or tone to 5 per cent corn mashes caused large yields of butanol t formed in place of butyric acid by *Cl. butylicum.* The stimulating e of asparagine was proportional to the amounts used up to a concentra of 0.4 g. of asparagine per 300 ml. of 5 per cent corn mash.

*Composition of Some Media.* In some of their research, Osburn his associates (1937) used a basal medium of the following com tion:

| | Per Cent |
|---|---|
| Glucose | 2 |
| Peptone | 0.7 |
| Difco yeast extract (powdered) | 0.2 |
| Dipotassium hydrogen phosphate | 0.1 |

glykke and his coworkers (1937) employed a medium containing
llowing ingredients:

| | Per Cent |
|---|---|
| Glucose | 3 (approx.) |
| Peptone | 0.5 |
| Asparagine | 0.1 |
| Dibasic ammonium phosphate | 0.07 |

ther peptone nor asparagine was satisfactory as the sole source of
gen.

kén and his associates (1952) studied the fermentation of glucose
yeast extract medium, in a medium containing yeast extract and
per cent $K_2HPO_4$, and in a synthetic medium which contained a
number of amino acids, glycocoll, ammonium acetate, $Na_2HPO_4$,
$PO_4$, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 2H_2O$, biotin, and water.
amounts of isopropanol and acetic acid produced by *Cl. butylicum*
erinck) Donker in the synthetic medium were greater than those
ced in the media containing yeast extract; however, the quantity of
ol was somewhat less. Freshly isolated strains of *Clostridium* pro-
l higher yields of acetic acid and butyric acid in the synthetic
um than in the media containing yeast extract.

**lustrial Production in Formosa.** A number of plants were con-
ed in Formosa during the years 1943 and 1944 for the industrial
ction of butanol, isopropanol, and acetone by a fermentation process
ich *Cl. toanum* Baba was used (Baba, 1952). The raw materials
included sugar cane, sugar sirup, raw sugars, and blackstrap molas-
The optimum sugar concentration for the economical production of
ts from mashes containing cane juice or sugar with molasses was
7.0 to 7.3 per cent (as glucose). Solvent yields of 30 to 33 per cent,
on the original sugars, were obtained after fermentation at 33 to
for 30 to 40 hr. The solvents were formed in ranges of 53 to 65 per
butanol, 19 to 44 per cent isopropanol, and 1 to 24 per cent acetone,
ature of the raw materials and the fermentation strongly influencing
atio of isopropanol to acetone. The solvents were separated by dis-
ion in continuous stills.

*oduction Studies.* Research was carried out over a period of years
nditions affecting the production of neutral solvents by *Cl. toanum*

e quality of the blackstrap molasses used greatly influenced the
s. Two types of molasses from sugar factories which used the defeca-
process for the clarification of raw sugar were satisfactory; two types
factories which used the carbonation process were unsuitable. How-
by mixing the suitable with the unsuitable types of molasses,

with a high proportion of the former, good yields were obtained (B 1945*a*).

Baba (1954*b*) reported on the solvent ratios obtained by the ferme tion of cane juice, or cane sugar and blackstrap molasses, to which 0.3 cent rice bran, 0.3 per cent ammonium sulfate, and 0.3 per cent calc carbonate were added, by *Cl. toanum* Baba at a temperature of 35°C. mashes contained 7 to 7.8 per cent sugar. The fermentation was compl in 31 to 37 hr. The solvents were composed of 50 to 58 per cent butano to 46 per cent isopropanol, and small amounts of acetone and ethanol. one series of experiments, the mean percentage ratio of the solvents 52.92 per cent butanol, 42.47 per cent isopropanol, 3.20 per cent etha and 1.41 per cent acetone.

During the fermentation, more than 1.5 times as much gas as neu solvents by weight were produced by *Cl. toanum* Baba (1953*a*).

Titrable acidity reached a maximum in 10 to 15 hr. and declined r idly up to 20 to 25 hr. The period in which the isopropanol increa coincided with that in which the acetone decreased in amount (Ba 1953*b*).

The addition of 0.5 per cent butanol to a sugar-containing mash at fifteenth hour of inoculation markedly arrested solvent production *Cl. toanum* Baba, while the addition of 1 per cent butanol "almost co pletely suppressed the fermentation" (Baba, 1954*c*). The addition 1 per cent acetone inhibited action slightly, whereas 1.5 per cent p vented solvent formation markedly.

The addition of 0.9 per cent isopropanol to a fermenting mash conta ing white sugar and molasses had only a slight effect on the fermentati but 1.5 per cent of this solvent reduced the yield of total solvents. T fermentation thus was only slightly affected by the isopropanol norma produced (up to 0.9 per cent) (Baba, 1953*c*).

**Isopropyl Alcohol from Wood Sugars.** The utilization of wo sugars in the production of butyl and isopropyl alcohols has been studi by Sjolander, Langlykke, and Peterson (1938).

The wood hydrolyzates, similar to some obtained in the Scholler pr ess, were treated to remove inhibitory substances. Excess calcium carb nate was added to neutralize the acidity of the hydrolyzates. The preci tate of calcium sulfate formed was removed by filtration, and the filtra was adjusted to a pH of approximately 10 with lime to cause precipitati of iron and copper. Again the medium was filtered. The filtrate w acidified to a pH of about 6.5, clarified with norite, and adjusted a pH of 6.

As a result of experimentation, a nutrient medium containing the f lowing constituents was used by Sjolander, Langlykke, and Peterson:

| | Per Cent |
|---|---|
| Malt sprouts | 2 |
| Dried whole liver | 0.25 |
| Dibasic ammonium phosphate | 0.05 |
| Treated wood sugar solution | As indicated |
| Calcium carbonate | 0.1 |

lcium carbonate was sterilized separately and added, using aseptic
utions, to the sterilized medium. The inoculated mashes were incu-
l at 37°C. for 5 days under anaerobic conditions.

e results of some experiments, using *Cl. butylicum*, are given in
e 86. An examination of this table indicates that more isopropyl

TABLE 86. PRODUCTS OF FERMENTATION BY *Clostridium butylicum* *

| rbohydrate source | Sugar as glucose, per cent |  | Neutral volatile products, per cent † | Distribution of neutral volatile products, per cent |  |  |  | Volatile acids,‡ mg./ml. |  |
|---|---|---|---|---|---|---|---|---|---|
|  | Original concen-tration | Fer-ment-ed |  | Butyl alco-hol | Ethyl alco-hol | Ace-tone | Iso-propyl alco-hol | Acetic | Butyric |
| ock 10 ........ | 3.15 | 92 | 34.6 | 59 | 9 | 4 | 28 | 2.4 | 1.3 |
| 11 .......... | 2.82 | 90 | 33.2 | 53 | 8 | 4 | 35 | 4.8 | 3.3 |
| se ........... | 2.90 | 99 | 29.8 | 70 | 7 | 3 | 20 | 1.0 | 1.1 |
| 11 (ether-ex-ted) ........ | 2.99 | 75 | 26.8 | 68 | 10 | 3 | 19 | 1.1 | 1.5 |
| se + acetate .. | 2.95 | 98 | 28.7 | 49 | 1 | 9 | 41 | 4.3 | 2.7 |

. O. Sjolander, A. F. Langlykke, and W. H. Peterson, *Ind. Eng. Chem.*, **30**: 1251–
(1938). Copyright 1938 by the American Chemical Society and reprinted by
ission of the copyright owner.

ased on the apparent sugar fermented.

olatile acids in uninoculated media (in milligrams per cubic centimeter):

|  | Acetic | Butyric |
|---|---|---|
| Hemlock 10 ................................ | 4.4 | 0 |
| Beech 11 ................................ | 9.0 | 0.2 |
| Beech 11 (ether-extracted) ................ | 0.4 | 0 |
| Glucose + acetate ........................ | 8.1 | 0.1 |

ol was formed from hemlock 10 and beech 11 wood sugars than from
se. It was assumed that the higher yields of isopropyl alcohol from
edia containing the wood hydrolyzates were due to the acetic acid
nts of the uninoculated media. These contents were reduced during
ermentation.

order to ascertain the correctness of the assumption that the
ased yields of isopropyl alcohol were due to the acetic acid present
e uninoculated media, Sjolander and his associates carried out two
riments. In one experiment beech 11 wood hydrolyzate was extracted
8 hr. continuously with ether. This ether-extracted medium was thus
y freed of volatile acids. Table 86 shows that the products formed
rmentation of this medium were similar quantitatively to those pro-
l from glucose. In the other experiment, a quantity of calcium ace-
equivalent to the acetic acid of the unextracted beech 11 wood
olyzate, was added to a medium containing glucose as the source of
r. The percentage of isopropyl alcohol produced was over twice that

produced in the glucose solution containing no calcium acetate, bu yield of butanol was considerably smaller.

**Solvents from Sulfite Liquors.** The production of neutral solv from waste sulfite liquors has been studied by Gfeller (1954), who f that of the *Clostridium* strains examined by him, *Cl. beijerinckii* wa best producer of butanol, acetone, and isopropanol. He used 0.5 per corn meal, 0.5 per cent pasteurized bakers' yeast (10 min. at 78°C.), per cent $(NH_4)_2HPO_4$, and 1.0 per cent $Na_2SO_4$ as nutrients and 0.1 cent $CaCO_3$ as a buffer as additives to the sulfite liquor. It was pos to ferment up to 67 per cent of the reducing substances present. Of fermentation products, about 60 per cent consisted of neutral solven

The addition of 50 to 75 mM. per liter of butyric acid to the u mented substrate more than doubled the yield of butanol (avera about 45 mM. per liter) and increased the yield of acetone and iso panol from 24 to 33 mM. per liter of substrate.

The addition of 75 mM. of acetic acid per liter of unfermented strate increased the production of acetone and isopropanol from 2 34 mM. per liter. There was a slight reduction in the yield of buta but increased amounts of butyric acid were formed.

**Effect of Neutralization with Sodium Bicarbonate.** Osburn and associates (1937) added sodium bicarbonate to fermenting glucose ma with the result that butyl alcohol and isopropyl alcohol production almost completely suppressed, the formation of isopropyl alcohol b inhibited more markedly than that of butanol. In the presence of r tively large concentrations of sodium bicarbonate, salts of acetic, buty formic, lactic, and pyruvic acids accumulated in the mash.

In carrying out the experiments, Osburn, Brown, and Werkman ad an 8 per cent solution of sodium bicarbonate to the inoculated glu mashes after the fermentations had become vigorous, usually after a 14 hr. In each case, the solution was added rapidly at first and t gradually until the weight of sodium bicarbonate added became equa the weight of the sugar being fermented. The results of these experim are shown in Table 87.

TABLE 87. FERMENTATION OF 2.0 PER CENT GLUCOSE BY *Clostridium butylicum*

(In presence of sodium bicarbonate, 0.7 per cent peptone, 0.2 per cent yeast ext 0.1 per cent dipotassium phosphate)

| Experiment no. | Products calculated per 100 mM. glucose fermented | | | | | | | Carbon dioxide, mM. | Hydrogen, mM. | Carbon recovered, per cent | Oxidation-reduction index |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Alcohols, mM. | | Acids, mM. | | | | | | | | |
| | Butyl | Isopropyl | Butyric | Acetic | Pyruvic | Lactic | Formic | | | | |
| 1 | 3.0 | 0 | 54.0 | 24.0 | 3.0 | 43.5 | 0 | 135.0 | 135.9 | 91.7 | 1.06 |
| 2 | 3.0 | 3.0 | 51.0 | 33.0 | 12.0 | 37.8 | 0 | 138.0 | 150.0 | 96.5 | 1.05 |
| 3 | 5.4 | 1.8 | 33.7 | 27.0 | 11.2 | 56.2 | 27.0 | 91.5 | 87.4 | 89.4 | 1.21 |
| 4 | 6.7 | 1.9 | 30.6 | 35.4 | 7.6 | 60.2 | 47.8 | 74.6 | 57.9 | 92.0 | 1.35 |

* O. L. Osburn, R. W. Brown, and C. H. Werkman, *Jour. Biol. Chem.*, 121: 685 (1937).

thylglyoxal was isolated from the fermenting mashes and identified.

**propyl Alcohol Production by B. technicus.** An organism iso- from *koji* rice and described in 1926 by Morikawa produced butyl sopropyl alcohols, acetic and butyric acids, carbon dioxide and hydro- and traces or small amounts of acetone as the principal end prod- from suitable nutrient carbohydrate media. Hydrolyzed *koji* rice; sirup; cerelose; mixtures of dextrins, maltose and glucose; or mix- of hydrolyzed *koji* rice with certain other sugars were readily fer- ed. Unhydrolyzed corn mashes, purified glucose or maltose, and strap molasses were poorly fermented with low yields. The optimum ntration of sugar was 10 to 13 per cent; the optimum reaction, pH 5 Good yields were obtained at temperatures of 28 to 37°C. Calcium nate stimulated the formation of neutral solvents.

**propyl Alcohol Production by Butyric Acid Anaerobes.** Some ric acid anaerobes produce isopropyl alcohol. However, the principal al solvent formed is butyl alcohol. Ethyl alcohol is formed in small tities, while variable amounts of acetone are produced. In Table 88 given data concerning the results of fermentations of glucose by ric acid anaerobes.

88. Fermentations Characterized by the Production of Isopropyl Alcohol*

| re | Glucose fermented,† per cent | Acidity 0.1 $N$ NaOH in 10 ml., ml. | Neutral volatile products based on glucose fermented, per cent | | | | |
|---|---|---|---|---|---|---|---|
| | | | Butyl alcohol | Ethyl alcohol | Iso-propyl alcohol | Acetone | Total |
| | 94.9 | 3.95 | 19.2 | 2.5 | 4.7 | 0.5 | 26.9 |
| | 94.9 | 3.70 | 22.2 | 2.2 | 4.4 | 0.7 | 29.5 |
| | 94.1 | 3.75 | 19.3 | 2.3 | 5.2 | 0.9 | 27.7 |
| | 78.0 | 5.30 | 17.8 | 2.1 | 4.5 | 0.6 | 25.0 |
| | 75.1 | 4.85 | 16.6 | 2.8 | 6.1 | 0.8 | 26.3 |
| | 94.8 | 4.30 | 15.7 | 2.4 | 3.2 | 1.6 | 22.9 |
| | 93.1 | 3.65 | 21.0 | 1.7 | 3.8 | 1.3 | 27.8 |
| | 94.5 | 3.30 | 17.1 | 2.4 | 3.8 | 3.7 | 27.0 |

. F. Langlykke, W. H. Peterson, and E. McCoy, *Jour. Bact.*, 29: 333–347 (1935).
he medium consisted of a double-strength yeast water ("the clear water t of fresh, starch-free yeast, 200 g. per liter of tap water") containing 2.5 per glucose with the reaction adjusted to pH 7.

**ields of End Products.** The quantities of end products formed from ose by *Cl. butylicum* in a series of laboratory experiments are shown able 89.

**fect of Adding Products to Fermenting Glucose Media.** The t of adding various substances to glucose mashes being fermented by *butylicum* has been studied recently by Langlykke and Fred; by glykke, Peterson, and Fred; by Osburn, Brown, and Werkman; and thers.

TABLE 89. FERMENTATION OF GLUCOSE BY *Clostridium butylicum* *

(Medium: 2 per cent solution with 0.7 per cent peptone, 0.2 per cent yeast ex
and 0.1 per cent dipotassium phosphate)

| Experiment no. | Products per 100 mM. fermented | | | | | | | Carbon recovered, per cent | Ox tion duc ind |
|---|---|---|---|---|---|---|---|---|---|
| | Alcohols, mM. | | | Acids, mM. | | Carbon dioxide, mM. | Hydrogen, mM. | | |
| | Butyl | Isopropyl | Ethyl | Butyric | Acetic | | | | |
| 1 | 57.6 | 15.1 | 2.9 | 15.1 | 9.1 | 166.5 | 75.8 | 87.0 | 0.8 |
| 2 | 66.6 | 15.1 | 0 | 6.1 | 21.2 | 197.0 | 72.7 | 95.0 | 1.0 |
| 3 | 58.6 | 12.1 | 0 | 17.2 | 17.2 | 203.5 | 77.6 | 96.3 | 1.0 |
| 4 | 54.6 | 15.1 | 2.9 | 18.2 | 12.1 | 200.0 | 121.2 | 93.5 | 0.9 |
| 5 | 65.5 | 13.8 | 2.3 | 13.8 | 10.3 | 189.5 | 86.2 | 95.6 | 0.9 |
| 6 | 50.2 | 18.0 | 0 | 14.5 | 20.3 | 207.0 | 111.1 | 93.5 | 1.0 |
| 7 | 54.1 | 18.0 | 0 | 15.5 | 12.9 | 222.0 | 107.0 | 96.2 | 1.0 |

* O. L. Osburn, R. W. Brown, and C. H. Werkman, *Jour. Biol. Chem.*, **121:**
(1937).

† Ratio of oxidized to reduced products; a perfect ratio = 1.0 [cf. Erb, Wood,
Werkman, *Jour. Bact.*, **31:** 595 (1936), for method of calculation].

In a normal fermentation of glucose by *Cl. butylicum*, acetone accun
lation in the fermenting medium before the formation of isopro
alcohol was observed (Langlykke et al., 1937). Acetone, a hydro
acceptor, when added to a fermenting glucose medium was converted
isopropyl alcohol. Acetic acid, when added, was transformed to isopro
alcohol. Acetaldehyde was converted to ethanol, butyric acid to butan
and acetylmethylcarbinol to 2:3-butyleneglycol under similar conditio
Pyruvic acid increased the yields of the products normally formed fr
glucose. Calcium or sodium pyruvate yielded acetic and butyric aci
Lactic acid was not fermented by *Cl. butylicum.*

Acetone and other hydrogen acceptors caused increased yields of is
propyl alcohol and acetone from glucose but decreased the yields
butyric acid and butanol. Since the added hydrogen acceptors utiliz
hydrogen, less was available for hydrogenation reactions (Langlykke a
Fred, 1937).

The addition of propionic acid to a medium containing yeast extra
and 2 per cent glucose (initially) resulted in increased amounts of acet
propionic, and butyric acids and *n*-propanol and decreased amounts
butanol and combined isopropanol and acetone, as compared with contr
containing no added propionic acid, by *Clostridium* strain 174 (Wik
and Zobrist, 1953). These researchers suggested that it is not impossib
that this microbe possesses the ability to metabolize propionic acid
accordance with the following scheme; however, they stated that th
reaction only partially explains how the propionic acid is used:

$$CH_3 \cdot CH_2 \cdot COOH + 2H_2O \rightarrow CH_3 \cdot COOH + 6H + CO_2$$

## Some References concerning the Butanol-Isopropanol Fermentation

H.: Über den Einfluss der Phosphatkonzentration auf die von *Clostridium utylicum* (Beij.) Donker bewirkte Butanol-Isopropanol-Gärung, *Schweiz. Zschr. llg. Path. Bakt.*, 13(5): 554–559 (1950).

—: Zur Kenntnis der von *Clostridium butylicum* (Beij.) Donker bewirkten utanol-Isopropanol Gärung mit besonderer Berücksichtigung des Einflusses von hosphat auf die Verteilung der Gärprodukt, Dissertation, ETH, Zurich, 1951.

T.: On the Industrial Production of Butanol, Isopropanol, and Acetone by a ermentation Process, *Bull. Fac. Eng., Hiroshima Univ.*, 1: 101–110 (April, 1952).

—: Studies on the Gas Formed during the Butanol and Isopropanol Fermenta- on of Sugar by *Clostridium toanum* Baba, *Bull. Fac. Eng., Hiroshima Univ.*, : 19–32 (February, 1953*a*).

—: On the Course of Solvent Formation in the Butanol and Isopropanol Fer- entation of Sugar by *Clostridium toanum* Baba, *Bull. Fac. Eng., Hiroshima niv.*, 2: 241–250 (1953*b*).

—: The Influence of Isopropanol on the Growth and Fermentation of Micro- rganisms. 1. The Effect of Isopropanol on the Fermentation of Sugar by *Clos- ridium toanum* Baba, *Bull. Fac. Eng., Hiroshima Univ.*, 2: 251–255 (1953*c*).

—: Studies on the Butanol, Isopropanol, and Acetone Fermentation of Black- trap Molasses by *Clostridium toanum* Baba, *Bull. Fac. Eng., Hiroshima Univ.*, : 147–152 (1954*a*).

—: On the Solvent Ratio in the Butanol-Isopropanol Fermentation of Sugar- ontaining Materials, *Bull. Fac. Eng., Hiroshima Univ.*, 3: 229–235 (1954*b*).

—: Influence of Butanol and Acetone on the Solvent Fermentation of Sugar by *lostridium toanum* Baba, *Bull. Fac. Eng., Hiroshima Univ.*, 3: 237–242 (1954*c*).

rinck, M. W.: *Verhandel. Akad. Wetenschappen Amsterdam, Afdeel. Natuur- unde*, Qde Sectie II, No. 10 (1893).

n, R. W., G. L. Stahly, and C. H. Werkman: Behavior of Butyric Acid-Butyl lcohol Bacteria toward Acetylmethylcarbinol and Asparagin, *Iowa State Coll. our. Sci.*, 12: 245–251 (1938).

ker, H. J. L.: Bijdrage tot de kennis der boterzuurbutylalcoholen acetongis- ngen, Thesis, Technische Hoogeschool, Delft, 1926.

, C. G.: A Biochemical Investigation of the Metabolic Activities of *Bacillus chnicus*, Thesis, Massachusetts Institute of Technology, Cambridge, Mass., 934.

ners, T.: Zersetzung von Kohlenhydraten durch *Granulobakterium butylicum* Beijerinck), *Tijdschr. Vergelijk. Geneeskunde*, 6: 33–39 (1920).

r, P.: Mikrobiologische Verwertung der Sulfitablaugen auf dem Garwege unter esonderer Berücksichtigung technisch verwendbarer Produkte, Dissertation, TH, Zurich, 1954.

ver, A. J.: Die bakteriellen Zuckervergärungen, *Ergeb. Enzymforsch.*, 4: 230–273 1935).

ykke, A. F., and E. B. Fred: *Jour. Bact.*, 33: 102 (1937).

—, W. H. Peterson, and E. B. Fred: Reductive Processes of *Clostridium butyli- um* and the Mechanism of Formation of Isopropyl Alcohol, *Jour. Bact.*, 34: 443– 53 (1937).

—, ———, and E. McCoy: Products from the Fermentation of Glucose and rabinose by Butyric Acid Anaerobes, *Jour. Bact.*, 29: 333–347 (1935).

kawa, K.: A New Butyl and Isopropyl Alcohol Fermentation, Doctoral Thesis, assachusetts Institute of Technology, Cambridge, Mass., 1926.

— and S. C. Prescott: A New Fermentation Yielding Butyl and Isopropyl Alco- ols, *Jour. Bact.*, 13: 58 (1927).

rn, O. L.: The Production of Butyl and Isopropyl Alcohols by Fermentation rocesses, *Iowa State Coll. Jour. Sci.*, 10: 97 (1935).

—, R. W. Brown, and C. H. Werkman: The Butyl Alcohol-Isopropyl Alcohol ermentation, *Jour. Biol. Chem.*, 121: 685–695 (1937).

———, ———, and ———: Dissimilation of Intermediary Compounds in the B Isopropyl Alcohol Fermentation, *Iowa State Coll. Jour. Sci.,* **12**: 275–284 (1

——— and C. H. Werkman: Utilization of Agricultural Wastes. II. Influen Nitrogenous Substrate on Production of Butyl and Isopropyl Alcohols by *tridium butylicum, Ind. Eng. Chem.,* **27**: 416–419 (1935).

Prescott, S. C., and K. Morikawa: Production of Butyl and Isopropyl Alcohols, Patent 1,933,683, Nov. 7, 1933.

Pringsheim, H. H.: Über den Ursprung des Fuselols und eine Alkohole bild Bakterienform, *Zentr. Bakt. Parasitenk.,* Abt. II, **15**: 330 (1906).

Sjolander, N. O., A. F. Langlykke, and W. H. Peterson: Butyl Alcohol Fermenta of Wood Sugar, *Ind. Eng. Chem.,* **30**: 1251–1255 (1938).

Stahly, G. L., O. L. Osburn, and C. H. Werkman: Quantitative Determinatio Acetone and Ethyl, Butyl, and Isopropyl Alcohols in Fermentation Liq *Analyst,* **59**: 319–325 (1934).

Van der Lek, L. B.: Onderzoekingen over de Butylalkoholgisting, Thesis, Techni Hoogeschool, Delft, 1930.

Wikén, T.: Die Bedeutung der radioaktiven und schweren Kohlenstoffisotope die Gärungsbiologie, *Schweiz. Brauerei-Rundschau,* Nr. **8**: 1–8 (1948).

——— und O. Richard: Biotin und *p*-Aminobenzoesäure als Wuchsstoffe für f isolierte Clostridium-Formen, *Physiologia Plantarum,* **5**: 510–527 (1952).

———, ———, und H. Aebi: Über die Vergärung der Glucose in einen synthetis Substrat durch Clostridium-Formen, *Schweiz. Zschr. allg. Path. Bakt.,* **15** 491–501 (1952).

——— und F. Zobrist: Über die Verwertung der Propionsäure durch Butylbakte *Schweiz. Zschr. allg. Path. Bakt.,* **16**(5): 836–845 (1953).

Zobrist, F.: Über die Abhangigkeit der Verteilung der Endprodukte der Clostridi Gärungen von der Art der Kohlenstoffquelle, *Schweiz. Zschr. allg. Path. B* **16**(5): 860–866 (1953).

## CHAPTER 15

# THE ACETONE-ETHANOL FERMENTATION

e acetone-ethanol fermentation differs from the acetone-butanol entation in several respects, namely, the type of organism used; the products, in particular with respect to the quantities of butyric acid utanol formed; the optimum pH; the use of calcium carbonate; and ime required for the completion of the fermentation.

hardinger (1905) is credited with being the first to discover acetone product of bacterial fermentation. The organism isolated and investi- l by him was designated as *Bacillus macerans.* Acetone, ethanol, acetic and formic acids were the principal end products obtained from ermentation of potatoes or potato starch media containing peptone calcium carbonate.

the suggestion of the Council of National Defense, Northrop under- research in an attempt to develop acetone through a fermentation ss. In 1919, Northrop, Ashe, and Senior isolated an organism, *cetoethylicum* (*B. acetoethylicus* Northrop et al.),[1] which produced one from starch. A careful study was made of the organism and the hemistry of the fermentation.

*acetoethylicus*, which is closely related, if not identical, to *B. rans*, was isolated from some old potatoes. It is a motile, sporeforming, 1-negative, facultative anaerobe. It grows well in a 2 per cent corn

90. QUANTITIES OF ACETONE AND ALCOHOL PRODUCED BY *Bacillus acetoethylicus* FROM VARIOUS SUBSTRATES *

ium: 2 per cent sugar; 0.5 per cent peptone, and 2 per cent $CaCO_3$; temperature, 37°C.; incubation period, 10 days)

| ubstance | Acetone, per cent | Alcohol, per cent | Substance | Acetone, per cent | Alcohol, per cent |
|---|---|---|---|---|---|
| tose ....... | 4– 5 | 22–24 | Dextrin .......... | 6– 7 | 14–16 |
| ose .......... | 6– 7 | 23–24 | Dextrose ........ | 9–10 | 22–23 |
| nose ........ | 6– 7 | 22–23 | Levulose ........ | 8–10 | 24–25 |
| ose ....... | 8–10 | 22–23 | Xylose .......... | 4– 5 | 18–20 |
| binose ..... | 6– 7 | 12–16 | Glycerol ........ | .... | 40–43 |
| m lactate .. | .... | ..... | Sucrose ......... | 8– 9 | 24–26 |
| .......... | 8–10 | 20–24 | | | |

---

H. Northrop, L. H. Ashe, and J. K. Senior, *Jour. Biol. Chem.*, 39: 1 (1919).

Bergey's Manual of Determinative Bacteriology, 7th ed., The Williams & Wil- Company, Baltimore, 1957.

medium containing calcium carbonate. Its optimum reaction for gr is a pH of 8 to 9; for fermentation, a pH of 6 to 8. It has an opti temperature of 40 to 43°C. Some of its spores will withstand boilin at least 20 min. Ethyl, propyl, and butyl alcohols; acetone; and f acid may be formed from suitable carbon-containing compounds. Tab shows some results of some fermentations.

**Raw Materials.** A large number of carbohydrate substances ma used for the fermentation. Corn, potatoes, and molasses are substa available in large quantities and at a reasonable cost. Hydrolyzed c cobs and oat and peanut hulls may serve as cheap sources of raw mat It is sometimes necessary to add peptone to a mash to supply the nitr required by the organism, as, for example, when the mash contains st alone. The use of concentrations of about 2 to 3 per cent of carbohy substances is customary.

**pH.** According to Northrop and his associates (1919), the optimum range for the growth of *B. acetoethylicus* (*B. acetoethylicum*) was 8 However, highest yields were obtained when the fermentation mash maintained at a pH of 6 to 8.

In the fermentation of xylose sirup, obtained from the hydrolysi corncobs, Peterson and his associates (1921) advised the use of an in reaction of pH 7.6 to 8.4 and the provision of sufficient calcium carbo to neutralize the acids as formed.

Arzberger, Peterson, and Fred (1920) have shown that the reactio the medium plays an important part in determining the relative qua ties of the various end products of the fermentation by *B. acetoethyli* An increase in the pH of the mash resulted in a decrease of alcohol duction and an increase in the volatile acid content. A marked reaction, a pH of 5.8 to 6, favored the production of acetone and decre the yield of acids. These results are indicated in Fig. 49.

Calcium carbonate is always used in this type of fermentation to bu

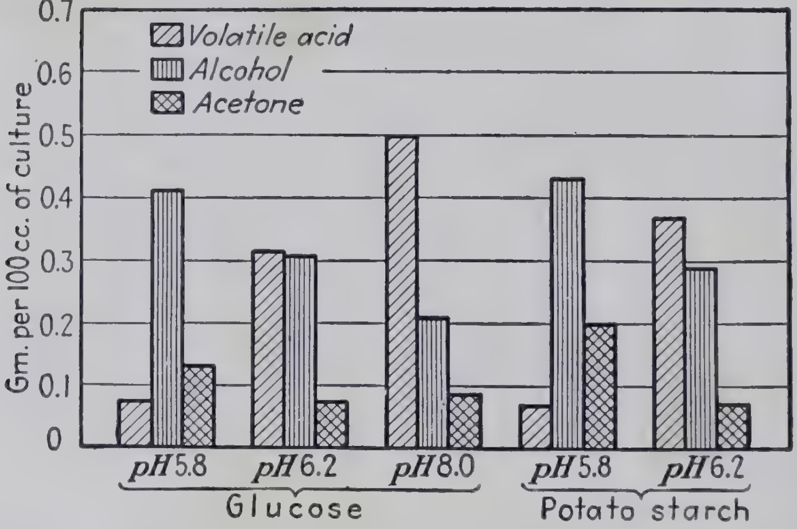


FIG. 49. The influence of varying the reactions of the medium on the produc fermentation. [*Courtesy of C. F. Arzberger, W. H. Peterson, and E. B. Fred, J Biol. Chem.*, **44**: 465 (1920).]

H. in sharp contrast to the acetone-butanol fermentation. A 2 per
oncentration is satisfactory in most cases.

**timum Temperature.** A temperature range of 40 to 43°C. is opti-
for fermentations produced by *B. acetoethylicus.*

**ration of the Fermentation.** The fermentation ordinarily requires
6 days. By using inert materials such as branches, coke, or corn-
to retain the slime which is formed during the fermentation and
contains bacteria and calcium carbonate, the time required for
rmentation may be considerably reduced. After the fermentation
een completed, the fermentation liquor is drawn off and a new mash
l to the vat containing the slime-coated inert material. This proc-
ay be repeated several times, but it is necessary to use precautions
vent contamination of the mash and to guard against sluggishness
culture.

TABLE 91. RATE OF FORMATION OF ACETONE AND ALCOHOL *

um: 10 g. of potato starch, 4 g. of peptone, 10 g. of calcium carbonate, and
500 ml. of water; temperature, 42°C.; time analyzed as noted)

| Culture no. 1 | | Culture no. 2 | | Culture no. 3 | | Average | | Ratio of alcohol to acetone | |
|---|---|---|---|---|---|---|---|---|---|
| Acetone, per cent | Alcohol, per cent | Acetone, per cent | Alcohol, per cent | Acetone, per cent | Alcohol, per cent | Acetone, per cent | Alcohol, per cent | By weight | By moles |
| 4.6 | 12.3 | 4.0 | 11.4 | 3.3 | 10.7 | 3.9 | 11.5 | 2.9 | 3.65 |
| 7.4 | 16.1 | 5.6 | 13.5 | 4.6 | 12.3 | 5.9 | 13.9 | 2.3 | 2.9 |
| 8.9 | 18.1 | 7.4 | 16.6 | 6.1 | 14.7 | 7.5 | 16.5 | 2.2 | 2.8 |
| 9.2 | 18.9 | 8.6 | 18.4 | 7.4 | 18.0 | 8.4 | 18.4 | 2.2 | 2.8 |
| 9.3 | 19.4 | 9.0 | 19.4 | 7.7 | 18.1 | 8.7 | 18.9 | 2.2 | 2.8 |
| 9.3 | 19.5 | 9.1 | 19.4 | 8.3 | 20.0 | 8.9 | 19.6 | 2.2 | 2.8 |
| 9.4 | 19.5 | 9.0 | 19.3 | 8.8 | 20.2 | 8.7 | 19.6 | 2.2 | 2.8 |

H. Northrop, L. H. Ashe, and J. K. Senior, *Jour. Biol. Chem.*, 39: 1 (1919).

**Fermentation of Corncobs.** Peterson, Fred, and Verhulst (1921)
d a method for hydrolyzing corncobs and fermenting the sugars thus
ced with *B. acetoethylicus.* The corncobs were hydrolyzed for 1 hr.
team pressure of 20 lb. The ratio of the weights of the water, corn-
and sulfuric acid used for the hydrolysis was 200:50:4. The hydroly-
obtained was neutralized with calcium hydroxide and pressed, and
sidue was washed. The sugar content of the mash was adjusted to a
cent concentration (as glucose). The reaction of the fermentation
naintained between 7.6 and 8.4 at the beginning. Sufficient calcium
nate was added to neutralize the acids formed. On the basis of
. of corncobs, the yield was 2.7 lb. of acetone, 6.8 lb. of ethanol, and
of volatile acids.

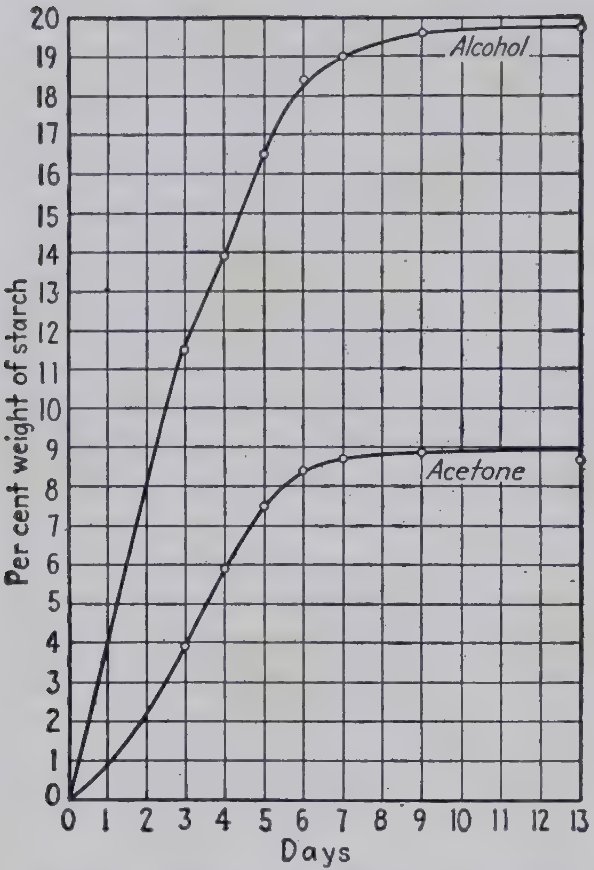


Fig. 50. Rate of formation of acetone and alcohol. [*Courtesy of J. H. North* *L. H. Ashe, and J. K. Senior, Jour. Biol. Chem.,* 39: 1 (1919).]

**The Fermentation of Oat and Peanut Hulls.** In 1923, Fred, Pe son, and Anderson reported that *B. acetoethylicus* (*B. acetoethylicu* produced acetone and ethanol from the sugars obtained by the hydrol of oat and peanut hulls. (These hulls are waste products obtained in manufacture of oatmeal and peanut butter.) The hulls were hydroly under a pressure of 15 lb. of steam for 2 hr. with 2 per cent sulfuric a The hydrolyzate was neutralized with milk of lime, and the sugars w extracted by pressing and washing. From oat hulls, as high as 26.5 cent of reducing sugars was obtained as glucose; from peanut hulls, per cent. The sugar concentration of the mash was adjusted to appro mately 3 per cent as glucose; calcium carbonate, peptone, and sodi phosphate were added, and the mash was inoculated with *B. acetoethyli* From 100 lb. of oat hulls, 7.2 lb. of ethanol, 3.9 lb. of acetone, and 1.4 of volatile acids were produced.

For further details of this fermentation consult some of the referer listed at the end of Chap. 13.

**Mechanism of the Ethanol-Acetone Fermentation.** Various theo have been proposed to explain the origin of the end products norma associated with this fermentation. The theories of Speakman (1925) a Bakonyi (1926) were the most important of the earlier ones. The int ested reader is referred to the publications of these scientists for deta

### References

See Chap. 13.

## CHAPTER 16

# THE LACTIC ACID BACTERIA

re are fairly large numbers of species of bacteria and some species
lds which possess the ability to form relatively significant quantities
tic acid from carbohydrates. Figure 51 shows some of the bacterial
s which produce lactic acid and which are of interest for one reason
other. The Gram-positive bacteria of the genera *Lactobacillus*,
*ococcus*, and *Leuconostoc* are of special importance in the food and
ntation industries. Their activities are illustrated in the following
ns of this book.

tic acid bacteria are of importance not only for the desirable reac-
which they catalyze but also for the undesirable activities which
promote. Table 92 presents some data concerning the reactions of
acid bacteria.

TABLE 92. DATA CONCERNING THE REACTIONS OF LACTIC ACID BACTERIA

Desirable Applications ✓

ough production (baking industry)
action production (fermentation
stry)
otics production
ermentations of cucumbers, olives,

starters
making
n manufacture
ated milks
ophilus
arian
ured
r
niss
urt

acid production
products manufacture
biological assays
no acids
mins of the B complex
kraut making
ge making (fermented types)
uces manufacture

Undesirable (Spoilage) Reactions ✓

Beer and other malt beverages
  Honey flavor
  Precipitates
  Souring
  Turbidities
Butanol-acetone fermentation
  Reduced yields
Curing pickles
  Ropiness
Distilling industries
  Reduced yields
Meat products
  Core greening
  Surface greening
  Turbidity
Milk souring
Orange-juice concentrates
  Buttermilk odor
  Souring
Slime formation
  On bologna, frankfurters, hams
  In sugar refineries
Vinegar-pickled products
  Turbidity
Wines
  Precipitates
  Souring
  Turbidities

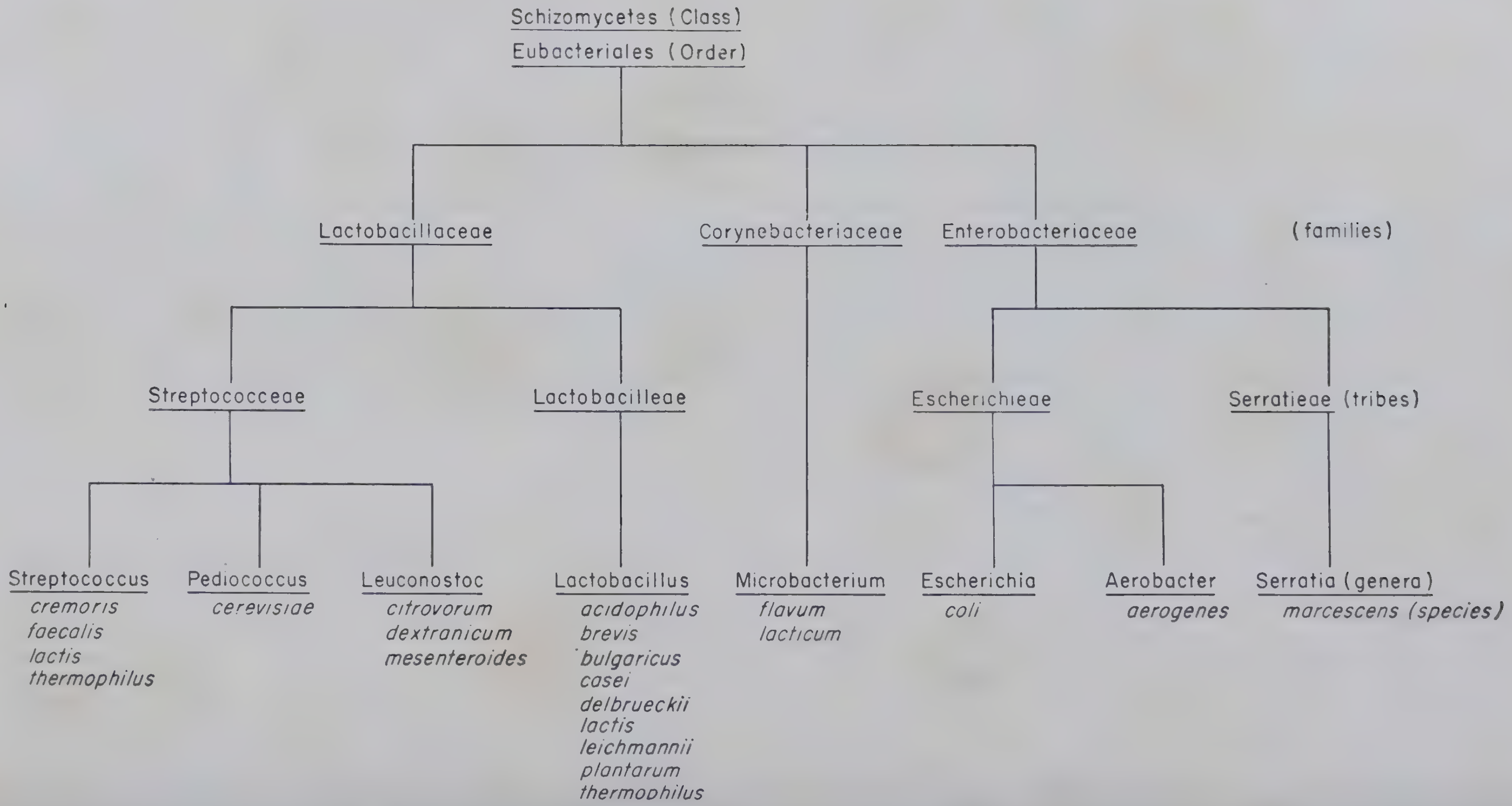

Schizomycetes (Class)
Eubacteriales (Order)
Lactobacillaceae
Corynebacteriaceae
Enterobacteriaceae
(families)
Streptococceae
Lactobacilleae
Escherichieae
Serratieae (tribes)
Streptococcus
cremoris
faecalis
lactis
thermophilus
Pediococcus
cerevisiae
Leuconostoc
citrovorum
dextranicum
mesenteroides
Lactobacillus
acidophilus
brevis
bulgaricus
casei
delbrueckii
lactis
leichmannii
plantarum
thermophilus
Microbacterium
flavum
lacticum
Escherichia
coli
Aerobacter
aerogenes
Serratia (genera)
marcescens (species)

FIG. 51. A classification of some of the lactic-acid-producing bacteria.

**tritive Requirements.** The nutritive requirements of the lactic
acteria, specifically members of the genera *Lactobacillus, Leuconos-*
nd *Streptococcus*, are rather complex. Various vitamins of the
nplex and certain amino acids are required for the growth of a
er of these microbes, in addition to the usual elements. In view of
exacting requirements, certain species of the genus *Lactobacillus*
other microbes) are used in assaying samples for a specific vitamin
ino acid.

nutritional requirements of the lactic acid bacteria have been
ved by Orla-Jensen (1919), Knight (1945), Peterson and Peterson
), Snell (1948, 1950, 1952), and others. Reviews relating to the
in requirements have been made by Shankman et al. (1947) and
ll, Bhandari, and Walker (1954, 1955). Special reference has been
to the amino acid requirements by Orla-Jensen, Otte, and Snog-
(1936); Dunn et al. (1947); and Bhandari, Russell, and Walker
).

composition of special culture media used in various studies may
nd described in a number of the papers cited above and in articles
derson and Ward (1949); Pederson and Albury (1950); Rogosa,
ell, and Wiseman (1951); Evans and Niven (1951); Tittsler et al.
); Cox and Briggs (1954); Niven, Buettner, and Evans (1954);
and Prouty (1954, 1955); Davis, Bisset, and Hale (1955); Deibel,
, and Niven (1955); and others. Information on culture media may
e found in *WADC Technical Report* 52-301 (1953).

st extract and malt sprouts may be used as sources of vitamins of
complex in media used for the isolation, growth, and maintenance of
ctic acid bacteria. Occasionally the addition of extra thiamin may
essary or desirable for the growth of some species.

**ssification.** A classification and descriptions of many species of
acid bacteria are to be found in "Bergey's Manual of Determina-
acteriology." Information relative to the classification of the lacto-
will be found in the publications of Orla-Jensen (1919); Tittsler,
and Rogosa (1947); Briggs (1953); Rogosa et al. (1953); Briggs
riggs (1954); Davis, Bisset, and Hale (1955); and elsewhere.
son (1949) described the genus *Pediococcus*, which contains species
ortance in the brewing industry, and discussed *P. cerevisiae* (1954).
one method of classification the true lactic acid bacteria may be
d into two groups. One group is made up of those bacteria which
rt carbohydrate materials to lactic acid as the principal end product.
*brueckii* is an example of this group. Kluyver and Donker have sug-
l that this group be called the "homofermentative" lactic acid bac-
in contrast to a second group of lactic acid bacteria which produces,
lition to lactic acid, volatile acids and carbon dioxide in quantity
or which they propose the name "heterofermentative." The latter
includes such bacteria as *L. lycopersici*, *L. mannitopoeus*, and *L.*
*ohil-aerogenes*. Lactic and acetic acids, ethanol, glycerol, and car-
ioxide are the main end products formed by these bacteria.
a second method of classification, the lactic-acid-forming bacteria

may be grouped, according to their significance to man, into organ of industrial importance, of which *L. delbrueckii, L. bulgaricus,* *Strept. lactis* are examples; organisms of possible therapeutic significa such as *L. acidophilus;* and organisms of sanitary significance, suc *E. coli.*

**Lactic Cultures.** A large amount of information relating to the p aration of lactic acid cultures will be found in the books by Elliker (19 Davis (1950), Hammer and Babel (1957), and others.

### Some References concerning the Lactic Acid Bacteria

Bhandari, R. R., C. Russell, and T. K. Walker: Studies of Lactic Acid Bacteria A ciated with Brewery Products. I. Identification of Types Isolated from Beer from Yeast, *Jour. Sci. Food Agr.,* No. 1, 1954, pp. 27–31.

———, ———, and ———: III. Amino-acid Requirements of 34 Organisms, J *Sci. Food Agr.,* No. 3, 1955, pp. 133–136.

Breed, R. S., E. G. D. Murray, A. P. Hitchens, et al.: "Bergey's Manual of Dete native Bacteriology," 7th ed., The Williams & Wilkins Company, Baltimore, 1

Briggs, M.: The Classification of Lactobacilli by Means of Physiological Tests, J *Gen. Microbiol.,* **9**: 234 (1953).

——— and C. A. E. Briggs: The Lactobacilli: A Review of the Literature with S cial Reference to Taxonomy, *Dairy Sci. Abstr.,* **16**: 252 (1954).

Collins, E. B.: Action of Bacteriophage on Mixed Strain Starter Cultures. I. Na and Characteristics of the "Nascent Phenomenon," *Jour. Dairy Sci.,* **35**(5): 3 380 (1952).

———: II. Relation to Acid Production of the Proportion of Resistant Bacte *Jour. Dairy Sci.,* **35**(5): 381–387 (1952).

———: III. Strain Dominance Due to the Action of Bacteriophage and Variation the Acid Production of Secondary Growth Bacteria, *Appl. Microbiol.,* **3**(3): 1 140 (1955).

———: IV. Domination among Strains of Lactic Streptococci, *Appl. Microb* **3**(3): 141–144 (1955).

———: V. Similarities among Strains of Lactic Streptococci in Commercially U Cultures and Use of a Whey Activity Test for Culture Selection and Rotat *Appl. Microbiol.,* **3**(3): 145–148 (1955).

Cox, C. P., and M. Briggs: Experiments on Growth Media for Lactobacilli, J *Appl. Bact.,* **17**: 18 (1954).

Davis, G. H. G.: The Classification of Lactobacilli from the Human Mouth, J *Gen. Microbiol.,* **13**: 481–493 (1955).

———, K. A. Bisset, and C. M. F. Hale: Correlation between Morphological Physiological Characters in the Classification of Members of the genus *Lac bacillus, Jour. Gen. Microbiol.,* **13**: 68 (1955).

Davis, J. G.: "A Dictionary of Dairying," Leonard Hill, Ltd., London, 1950.

Deibel, R. H., J. B. Evans, and C. F. Niven, Jr.: Thiamin Deficiency of Media C taining Yeast Extract for Growth of Heterofermentative Lactobacilli, *Bacte logical Proceedings* 1955, *Society of American Bacteriologists,* 1955, p. 48.

Dunn, M. S., S. Shankman, M. N. Camien, and H. Block: The Amino Acid Requ ments of 23 Lactic Acid Bacteria, *Jour. Biol. Chem.,* **168**: 1 (1947).

Elliker, P. R.: "Practical Dairy Bacteriology," McGraw-Hill Book Company, I New York, 1949.

Evans, J. B., and C. F. Niven, Jr.: Nutrition of Heterofermentative Lactobac That Cause Greening of Cured Meat Products, *Jour. Bact.,* **62**: 599 (1951).

Glenn, W. E., and C. C. Prouty: Bacteriological Studies of Cultured Butterm II. *Leuconostoc citrovorum* and *Streptococcus cremoris* as Associated with and Acetylmethylcarbinol-plus Biacetyl Levels, *Jour. Dairy Sci.,* **37**(9): 11 1133 (1954).

——— and ———: Bacteriological Studies of Cultured Buttermilk. III. The Eff

Additions of Citric Acid, Sodium Citrate and Lactic Acid on the Progressive anges in the Numbers of *Leuconostoc citrovorum* and *Streptococcus cremoris* Associated with Acetylmethylcarbinol Plus Biacetyl and pH Levels, *Appl. crobiol.*, **3**(6): 317–320 (1955).

R., and B. Krasse: Classification of Oral Strains of Lactobacilli, *Acta Pathol. crobiol. Scand.*, **32**: 539 (1953).

r, B. W., and F. J. Babel: "Dairy Bacteriology," 4th ed., John Wiley & Sons, ., New York, 1957.

B. C. J. G.: "Growth Factors in Microbiology—Some Wider Aspects of tritional Studies with Microorganisms, Vitamins and Hormones," Academic ss, Inc., New York, 1945, Vol. III, pp. 105–228.

C. F., Jr.: Significance of the Lactic Acid Bacteria in the Meat Industry, *ceedings of the 4th Research Conference*, American Meat Institute, Council Research, University of Chicago, Chicago, March 20, 21, 1952, pp. 31–37.

L. G. Buetttner, and J. B. Evans: Thermal Tolerance Studies on the terofermentative Lactobacilli That Cause Greening of Cured Meat Products, *pl. Microbiol.*, **2**(1): 26–29 (1954).

nsen, S.: The Lactic Acid Bacteria, *Mém. acad. roy. sci. Danemark Sect. sci.*, e Ser., **5**: 81–196 (1919).

N. C. Otte, and A. Snog-Kjaer: Der Vitaminbedarf der Milchsäurebakterien, *ntr. Bakt. Parasitenk.*, Abt. II, **94**: 434–447 (1936).

n, C. S.: The Genus Pediococcus, *Bact. Revs.*, **13**(4): 225–232 (1949).

and M. N. Albury: Effect of Temperature upon Bacteriological and Chemical anges in Fermenting Cucumbers, *N.Y. State Agr. Expt. Sta., Bull.* 744, 1950.

M. N. Albury, and R. S. Breed: *Pediococcus cerevisiae*, the Beer Sarcina, *llerstein Labs. Communs.*, **17**(56): 7–17 (1954).

and L. Ward: The Effect of Salt upon the Bacteriological and Chemical anges in Fermenting Cucumbers, *N.Y. State Agr. Expt. Sta., Tech. Bull.* 288, 49.

n, W. H., and M. S. Peterson: Relation of Bacteria to Vitamins and Other owth Factors, *Bact. Revs.*, **9**: 49–109 (1945).

. C. C., and W. E. Glenn: Bacteriological Studies of Cultured Buttermilk. A Limited Plate Culture Method for the Differentiation and Enumeration of *uconostoc citrovorum*, *Proc. Western Div. Am. Dairy Sci. Assoc. 35th Ann. eting*, 1954.

. M., J. A. Mitchell, and R. F. Wiseman: A Selective Medium for the Isoion and Enumeration of Oral Lactobacilli, *Jour. Dental Research*, **30**: 682 51).

R. F. Wiseman, J. A. Mitchell, M. N. Disraely, and A. J. Beaman: Spes Differentiation of Oral Lactobacilli from Man Including Descriptions of *tobacillus salivarius* nov. spec. and *Lactobacillus cellobiosus* nov. spec., *Jour. ct.*, **65**: 681 (1953).

, C., R. R. Bhandari, and T. K. Walker: Vitamin Requirements of Thirtyr Lactic Acid Bacteria Associated with Brewery Products, *J. Gen. Microbiol.*, (3): 371–376 (1954).

———, and ———: Studies on Lactic Acid Bacteria Associated with Brewery oducts. IV. Interrelationships in Requirements for Purines, Folic Acid and Aminobenzoic Acid, *Jour. Sci. Food Agr.*, **6**(10): 633–636 (1955).

man, S., M. N. Camien, H. Block, R. B. Merrifield, and M. S. Dunn: Vitamin quirements of 23 Lactic Acid Bacteria, *Jour. Biol. Chem.*, **168**: 23 (1947).

E. E.: Nutritional Requirements of the Lactic Acid Bacteria, *Wallerstein Labs. mmuns.*, **11**: 81–104 (1948).

: "Microbiological Methods in Vitamin Research," Vol. I, pp. 327–421, of itamin Methods," Academic Press, Inc., New York, 1950.

r, R. P., D. S. Geib, and M. Rogosa: Taxonomy of the Genus *Lactobacillus*, th Special Reference to Correlations of Differential Characteristics, *Jour. Bact.*, : 12–13 (1947).

, C. S. Pederson, E. E. Snell, D. Hendlin, and C. F. Niven, Jr.: Symposium on e Lactic Acid Bacteria, *Bact. Revs.*, **16**(4): 227–260 (1952).

## CHAPTER 17

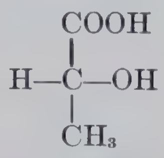
# THE PRODUCTION OF LACTIC ACID BY FERMENTATI

Lactic acid, or alpha-hydroxypropionic acid ($CH_3 \cdot CHOH \cdot COOH$
an unnamed component of soured milk must have been known in hu
experience since the days when man first had his flocks and herds.
true nature was discovered by Scheele, who isolated and identified i
the principal acid in sour milk in 1780.

Lactic acid was first discovered as a fermentation product by Blon
in 1847. It was investigated by Pasteur as one of his first microbiolog
problems. Schultze (1868) demonstrated the presence of lactic acid
teria in yeast cultures of distilleries. But it was not until the year
that lactic acid bacteria were isolated in pure cultures, Lister having
lated *Streptococcus lactis*. During this same period Delbrück was end
oring to determine the most favorable temperature for lactic acid
mentation in distilleries. He concluded that relatively high temperat
favored high yields of lactic acid.

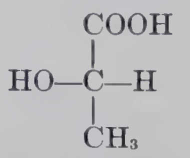
Avery, of Littleton, Mass., was the first person (1881) to produce la
acid successfully on a commercial basis. At that time an effort was b
made to substitute calcium lactate for the tartrates then being use
baking powders. The attempt at replacement was unsuccessful, but m
new uses for the acid were found. Since 1881 the production of lactic
by fermentation has become a very important industry. Lactic aci
now produced commercially from corn sugar, molasses, and whey.

**Forms of Lactic Acid.** Lactic acid occurs in two optically active fo
the D(−)- and the L(+)-isomers, and in one optically inactive f
DL-. The formulas for the optically active isomers are shown below.



$$\begin{array}{c} COOH \\ | \\ H{-}C{-}OH \\ | \\ CH_3 \end{array}$$

D(−)-Lactic acid



$$\begin{array}{c} COOH \\ | \\ HO{-}C{-}H \\ | \\ CH_3 \end{array}$$

L(+)-Lactic acid

D(−)-Lactic acid possesses the D-configuration and is levorotat
L(+)-lactic acid possesses the L-configuration and is dextrorotatory.
salts of D(−)-lactic acid are dextrorotatory; those of L(+)-lactic
are levorotatory.

The lactic acid formed during fermentation is usually the DL-
(a racemic mixture). Pederson et al. (1926) and later Tatum and as
ates (1932) showed that *Clostridium acetobutylicum* caused lactic
bacteria, which usually formed optically active lactic acid, to produc

ve form. It was suggested by Tatum et al. that racemization might ught about by an enzyme system elaborated by *Cl. acetobutylicum l. butylicum.*

agiri and Kitahara (1935–1938) demonstrated that the conversion ically active lactic acids to the optically inactive form was due to yme (racemase). This enzyme is produced by a number of bacteria, ing some lactic acid bacteria.

ahara and Obayashi (1955) found that nicotinic acid was essential e growth and fermentation of DL-lactic acid bacteria and that the of racemase activity coincided with the concentration of nicotinic n the medium. The DL-forming lactic acid bacteria used by them strains of *Lactobacillus plantarum* and one strain each of *L. arabi- L. japonicus, L. brevis,* and *Pediococcus hennebergi*) required nico- acid as a growth factor. Deficiency of nicotinic acid in the produc- edium resulted in an increase in optical activity. Measurement of ptical activity indicated whether the organism produced D-lactic r L-lactic acid.

ring a normal fermentation, the pyruvic acid formed as an interme- is acted on by either a D-lactic dehydrogenase or an L-lactic dehy- nase (Table 93) to produce D(−)-lactic acid or L(+)-lactic acid hara, Obayashi, and Fukui, 1952).

93. SOME DATA CONCERNING LACTIC DEHYDROGENASES WHICH ARE PRODUCED BY LACTIC ACID BACTERIA

| teria producing the dehydrogenase | Form of lactic acid produced | Linked with | References |
|---|---|---|---|
| ermentative lactic acid eria and *Escherichia* .................... | L(+)- | DPN | Kaufman et al.: *Jour. Biol. Chem.*, **192**: 301 (1951).<br>Korkes et al.: *Nature,* **166**: 439 (1950).<br>——— et al.: *Jour. Biol. Chem.,* **193**: 721 (1951). |
| fermentative lactic acid eria, *Leuconostoc mes- roides* and *Lactobacil- rabinosus* ............ | D(−)- | DPN | DeMoss et al.: *Jour. Bact.,* **62**: 499 (1951).<br>Kaufman et al.: *Jour. Biol. Chem.,* **192**: 301 (1951). |
| *brueckii* ............. | L(+)- | Flavin coenzyme | Hager et al.: *Federation Proc.,* **13**: 734 (1954).<br>Lipmann: *Jour. Biol. Chem.,* **134**: 463 (1940). |

## THE COMMERCIAL PRODUCTION OF LACTIC ACID

**ne General Considerations.** *Organisms Used.* The organisms that be used for the production of lactic acid by fermentation are *Lacto-*

*bacillus delbrueckii, L. bulgaricus, L. pentosus, L. casei, L. leichm* and *Streptococcus lactis.* All these organisms are homofermentative.

Andersen and Werkman (1943) isolated and studied a sporefor bacillus that produced large amounts of the dextrorotatory lactic The name *Bacillus dextrolacticus* was proposed for this organism.

The type of organism selected for a fermentation depends prim upon the carbohydrate being fermented and the temperature to be *L. bulgaricus, L. casei,* or *Strept. lactis* may be used to ferment mil whey, *L. bulgaricus* being favored. In the fermentation of dextro maltose *L. delbrueckii, L. pentosus, L. leichmannii,* or *L. bulgaricus* be used. Frequently *L. delbrueckii* is used with another lactic acid ducer, such as *L. bulgaricus* or *Strept. lactis,* to ferment hydrol starches.

*Carbohydrates Suitable for Utilization.* A large number of ca hydrates may be utilized for lactic acid production. The acid is gener produced from glucose, sucrose, or lactose. Starches, corn and po especially, may be hydrolyzed by enzymes, or by acids (preferably furic acid) to maltose and glucose. Xylose is fermented by *L. pe aceticus* to yield lactic acid and acetic acid, chiefly. Molasses and w are generally low-priced sources of sugars for the fermentation. Sr and Claborn (1939) have estimated that 27 billion lb. of lactos obtained from skim milk, buttermilk, and whey annually. Of this qu tity, a large percentage could be used in lactic acid production. Su waste liquor and Jerusalem artichokes are potential sources of la acid. The choice of carbohydrate used will depend upon its availabi its fermentability, with or without preliminary treatment; and its c In this country, corn sugar, molasses, and whey are much used; in G many, potato starch.

*Temperature of the Fermentation.* The lactic acid fermentatio carried out at comparatively high temperatures. In fermentations u *L. delbrueckii* a temperature of 45°C., or higher, may be maintai *L. bulgaricus* may be incubated at 45 to 50°C.; *L. pentosus, L. case Strept. lactis,* at about 30°C. The optimum temperature should be de mined experimentally for each type of fermentation.

*Concentration of Sugar.* The sugar in mashes is normally adjuste a concentration of 5 to 20 per cent, depending on the nature of the material and the conditions of the process.

*Oxygen Relationship.* The bacteria used to produce lactic acid ind trially are usually microaerophilic or anaerobic in nature. *Strept. la* is listed as a facultative aerobe.

*pH.* The fermentation proceeds best when the pH is on the acid of neutrality. Owing to the addition of calcium carbonate, calc hydroxide, or some other neutralizing agent to the fermentation m the pH tends to approach neutrality. The pH may be maintained a constant value by continuous control, with the use of ammonia, amm nium carbonate, or mixtures of sodium, potassium, and ammonium car nate (Kempe et al., 1950) as the neutralizing agents.

Continuous control of pH in the lactic acid fermentation is adv ıs, since yields and rates of production may be increased thereby. rd et al. (1948) found that the optimum pH for lactic acid produc- om pretreated sulfite waste liquor was 5.8.

npe and associates (1950) studied the effect of controlled pH on the and rate of lactic acid production, using wheat grits, saccharified enzymes of *Aspergillus oryzae*, with and without added nutrients, rmented with *L. delbrueckii* NRRL B-445 at 45°C. When the mash ned plain saccharified wheat grits only, they found that the maxi- yield occurred at pH 6 and that lactic acid was produced 5 times rapidly at this pH than at 4.5. When, however, the saccharified grits were fortified with yeast extract and mineral salts, the maxi- yield occurred at pH 4.6 and decreased slowly as the pH was raised Also, the rate of acid formation in the supplemented mash at 6 was about equal to that of the plain mash at 6, but the yield ower.

se studies demonstrate the value of controlled pH. Undoubtedly, vements in yields will result when pH control is perfected for the ercial process.

he lactic acid were not neutralized, the lactic acid bacteria would able to tolerate the high acidity developed and the fermentation not continue to completion.

cium (or zinc) hydroxide or carbonate may be added either at the ning of the fermentation or intermittently as the fermentation sses. Fred, Peterson, and Davenport suggested that the preliminary uction of a neutralizing agent was as efficacious as intermittent in- tion from the point of view of the speed and completeness of the rsion of xylose to lactic acid.

*wth Factors for Lactic Acid Bacteria.* Certain growth factors r to be essential for certain lactic acid bacteria. Orla-Jensen and low workers (1936) reported that riboflavin and at least one other ator" were required by certain lactic acid bacteria for normal pment. Wood and his associates (1937) confirmed their findings in t to riboflavin.

il, Strong, and Peterson (1937) described the preparation of an concentrate from liver extract, which was essential for the normal of the species of lactic acid bacteria investigated by them. It has hown that this active substance was pantothenic acid (Snell et al., 1939).

otinic acid stimulated growth and acid production by some lactic acteria.

*entosus* 124–2 requires biotin, pantothenic acid, and nicotinic acid, ling to Krueger and Peterson (1948).

further information concerning this important subject, the reader rred to the foregoing citations and others listed at the end of the er.

*essory Nutrients in the Fermentation of Molasses.* Stiles and (1938) have shown that good yields of lactic acid and a short fer- tion period result from the addition of such accessory nutrients as malt sprouts, steep water, and thin grain residue to blackstrap mo The yield and fermentation time depend on the kind, quantity, and bination of the accessory nutrients. It was suggested that the acc nutrients supplied soluble organic nitrogen and stimulatory subs that were useful biologically.

*Duration of Fermentation.* A fermentation is usually compl 1 to 6 days.

*Yields.* Yields of 85 to 90 per cent on the basis of the sugar ferm are not uncommon in controlled processes. Higher yields have obtained occasionally. Tatum and Peterson (1935) have reported a of 100.5 g. of L(+)-lactic acid from 100 g. of glucose.

*Grades of Lactic Acid.* There are at least four principal grad lactic acid. These include a crude or technical grade of 22, 44, and 8 cent strengths, edible lactic acid of 50 and 80 per cent strengths, pl grade lactic acid of 50 and 80 per cent strengths, and U.S.P.-grade acid of 75 and 85 per cent strengths (Peckham, 1944).

Standards may be set up by the consumers, the requirements de ing on the use to be made of the lactic acid. Color, flavor, and odo important, in addition to the ash content. For example, lactic acid in the manufacture of transparent phenolic resins must be of high pu The chloride, sulfate, and ash contents must be very low, especially iron.

## LACTIC ACID FROM WHEY

Lactic acid has been produced commercially from lactose since the 1936 at Norwich, N.Y., in a plant operated by the Sheffield By-Pro Company (a subsidiary of the Sheffield Farms, which in turn is a sidiary of the National Dairy Products Corporation of New York). plant has a capacity for 10,000 lb. of 22 per cent lactic acid per Technical and U.S.P. grades of lactic acid, calcium lactate, and so lactate are manufactured from whey (Olive, 1936).

The utilization of casein whey has always been a problem. It ma dried and used as a feed for cattle and poultry, or the lactose and albu may be recovered from it. Now, it may be used in the commercial duction of lactic acid.

Surplus milk and skim milk from other sources may be used in manufacture of lactic acid. The cream is removed from the milk the casein precipitated by the use of lactic acid or hydrochloric acid, former acid being preferred, for it may be recovered subsequently. whey, known as casein whey, which is the product left after the separa of the cream and casein from the milk, contains lactalbumin (a prot approximately 4.6 per cent lactose, vitamin G, mineral salts, and w This medium serves as the nutrient substrate in the manufactur lactic acid.

The commercial process for the production of lactic acid from the tose of whey is based on the researches of Rogers and his associat the Bureau of Animal Industry, U.S. Department of Agriculture. Cl l his associates of the Sheffield By-Products Company and others esearch laboratories of the National Dairy Products Corporation imore were largely responsible for making the process a commercial is process has been described by Olive and by Burton (1937). The tions that follow are based on the articles by L. V. Burton.

**Process in Brief.** Pasteurized whey is inoculated with a starter ing *Lactobacillus bulgaricus.* During the fermentation the lactic oduced from lactose is neutralized intermittently with lime. At of the fermentation, the lactalbumin is coagulated by heat. After ing the coagulated lactalbumin to settle, the solution of calcium is decanted off, filtered, treated with decolorizing carbon and filter ltered, and evaporated. Crystallization follows. The crystals are , perhaps further purified, and dried and may be sold as calcium or converted to lactic acid. Various procedures are followed in ing the different grades of lactates and lactic acid.

**aration of the Starter.** The starter is prepared as follows: A f sterilized skim milk is inoculated with a culture of *L. bulgaricus,* ing also a yeast that causes the fermentation to become more s. After incubation for 24 hr. at a temperature of 43°C. (110°F.), tents of the bottle are placed in a 40-qt. jug containing pasteurized ilk. The contents of the jug are dumped, after incubation at a ature of 43°C. for 24 hr., into a glass-lined steel tank containing l. of pasteurized whey. Following incubation at 43°C. for 24 hr., rter is added to the main fermentation tank (see Fig. 52).

**Fermentation Tank.** This tank is constructed of wood and has a y for 5,000 gal. of whey. In the bottom of the fermentor is a ted brass pipe, through which steam may be introduced to warm sh to the temperature desired for the fermentation or for the ent coagulation of lactalbumin. The tank is provided with a ical stirrer and a 4-in. brass dip pipe, which may be raised or l in order to decant the clear solution of calcium lactate, which es after the coagulation of lactalbumin. Before use, the tank is l. treated with a chemical agent to destroy microorganisms, and ently rinsed with pure water. An outlet is located at the bottom ank for use in connection with its cleaning.

**Fermentation.** The inoculated whey is maintained at a temperature 43°C. until the end of the fermentation, which is usually complete t 42 hr. Lime, $Ca(OH)_2$, in the form of a slurry, is added to the ting mash every 6 hr. to keep the acidity of the mash below 0.6 per By neutralizing the lactic acid with lime, the fermentation time is and the yields are higher, for under these circumstances the a are not inhibited by the acid that they produce. At the completion fermentation, the mash is neutralized to 0.1 per cent lactic acid.

**ation of the Fermented Mash.** The fermented medium, which pale-green color, is heated to 96°C. (205°F.) to coagulate the umin. The coagulated albumin is permitted to settle. Only a short necessary, however, for in about 10 min. the supernatant liquor e decanted.

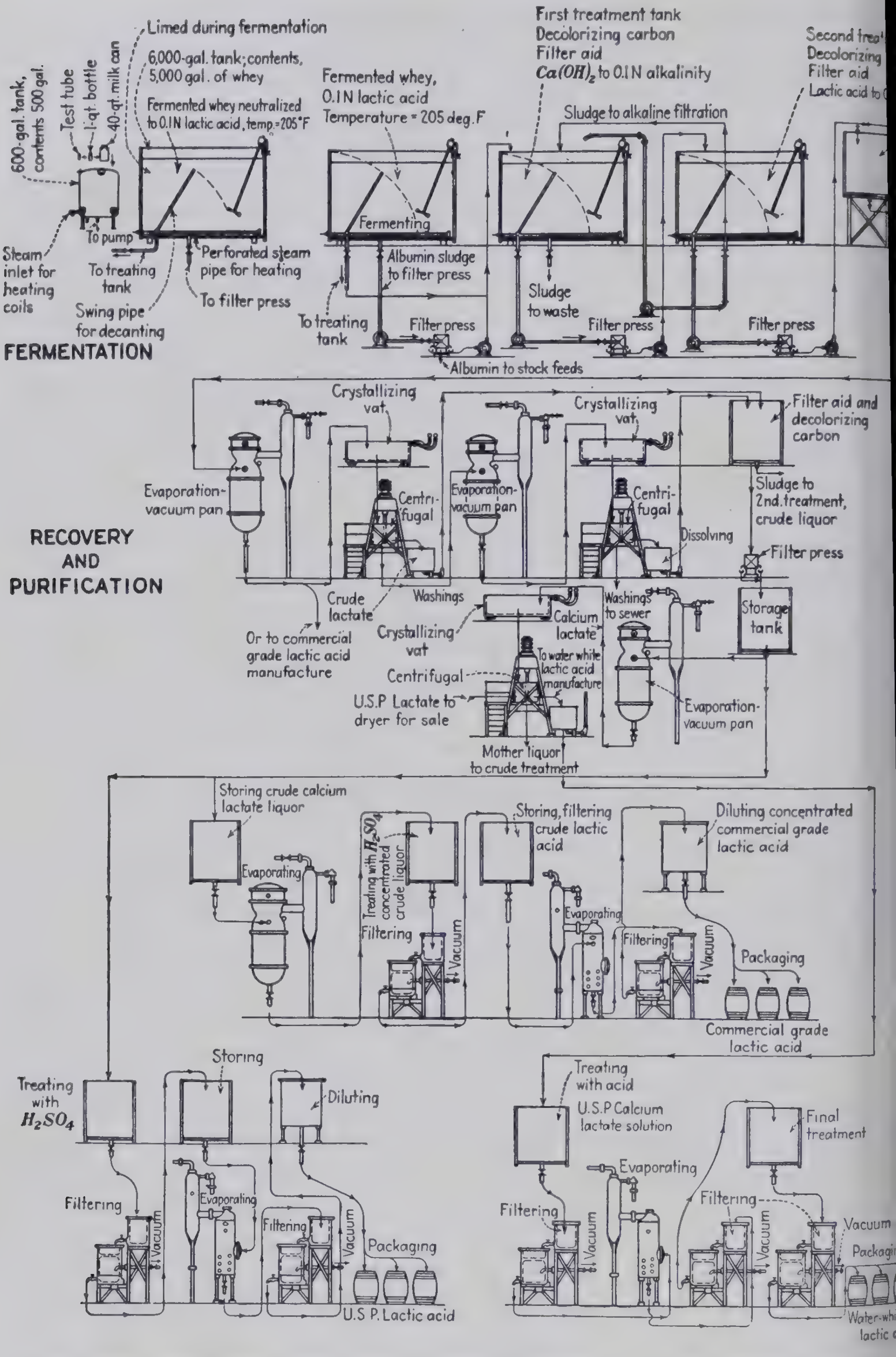

Limed during fermentation
600-gal. tank, contents 500 gal.
Test tube
1-qt. bottle
40-qt. milk can
6,000-gal. tank; contents, 5,000 gal. of whey
Fermented whey neutralized to 0.1N lactic acid, temp.=205°F
Fermented whey, 0.1N lactic acid Temperature = 205 deg. F
First treatment tank
Decolorizing carbon
Filter aid
Ca(OH)2 to 0.1N alkalinity
Second trea
Decolorizing
Filter aid
Lactic acid to
Sludge to alkaline filtration
Steam inlet for heating coils
To pump
To treating tank
Swing pipe for decanting
Perforated steam pipe for heating
To filter press
Fermenting
Albumin sludge to filter press
To treating tank
Filter press
Sludge to waste
Filter press
Filter press
Albumin to stock feeds
FERMENTATION
Crystallizing vat
Crystallizing vat
Filter aid and decolorizing carbon
Evaporation-vacuum pan
Centrifugal
Evaporation-vacuum pan
Centrifugal
Sludge to 2nd. treatment, crude liquor
Dissolving
Filter press
RECOVERY AND PURIFICATION
Crude lactate
Washings
Washings to sewer
Storage tank
Or to commercial grade lactic acid manufacture
Crystallizing vat
Calcium lactate
To water white lactic acid manufacture
Centrifugal
U.S.P Lactate to dryer for sale
Evaporation-vacuum pan
Mother liquor to crude treatment
Storing crude calcium lactate liquor
Evaporating
Treating with H2SO4 concentrated crude liquor
Filtering
Vacuum
Storing, filtering crude lactic acid
Evaporating
Filtering
Vacuum
Diluting concentrated commercial grade lactic acid
Packaging
Commercial grade lactic acid
Treating with H2SO4
Storing
Diluting
Filtering
Vacuum
Evaporating
Filtering
Vacuum
Packaging
U.S.P. Lactic acid
Treating with acid
U.S.P Calcium lactate solution
Evaporating
Final treatment
Filtering
Vacuum
Filtering
Vacuum
Vacuum
Packagi
Water-whi
lactic

Fig. 52. Lactic acid from casein whey. (*Courtesy of L. V. Burton, Food Ind*

The supernatant liquor is withdrawn through the decanting pip forced, by means of a centrifugal pump, through a plate-and-frame of filter press to a storage tank. Here it is treated with lime to adjus alkalinity to 0.1 per cent. A filter aid, such as diatomaceous earth, s decolorizing carbon (Norit) are added. The mixture is throughly and then the contents are permitted to stand. After approximately n. the clear supernatant is decanted off and pumped through a filter The sludge is discarded. The clear solution, pumped to a clean n tank, is acidified to 0.05 per cent acid with lactic acid. Filter aid ecolorizing carbon are again added, settling is permitted, and the natant liquor is decanted and filtered. Sludge from this second treat- may be used to treat a fresh lot of calcium lactate solution.

**aporation, Crystallization, and Washing.** The filtrate, or crude r, may be stored in a clean wooden tank (Fig. 52), or it may be con- ated in an evaporator, under a vacuum of 25 in., to a concentration °Bé and then pumped to crystallizing pans (equipped with casters cilitate their being moved about) located on a gallery above the containing the evaporators. The crystallizing vats each have a ity for 300 gal. Each is provided with a water jacket, and the inner which comes in contact with the calcium lactate, has a lining of ess steel. Cooling water is passed through the jackets of the pans. um lactate crystallizes out after 10 to 12 hr. at 10 to 15.6°C. (50 °F.). The pans are pushed to chutes and the crystallized materials led down them to the baskets of Hepworth centrifugals. The ts are spun. The mother liquor, which passes off, is reserved for er purification treatment. The crystals of calcium lactate that in are washed with water, while the baskets are still spinning. The water thus obtained is evaporated to 13.5°Bé and recrystallized. he new crop of crystals is centrifuged and washed. The wash water gain evaporated, crystallization takes place, and the crystals are ed, the wash water being discarded this time.

om this series of treatments, three sets of crystals have resulted. e are combined, placed in a glass-lined tank, and dissolved in a small nt of water at 66°C. (150°F.). Norit and filter aid are mixed with rude calcium lactate solution, the Norit for improving the color and ilter aid for removing finely suspended particles during filtration. supernatant is pumped to a filter press, while the sludge is returned e second treatment of the crude liquor. The thus refined liquor may oncentrated to 11.5°Bé and then placed in vats for crystallization. tallization proceeds slowly, with the result that the crystals are r. The crystals are washed, the washings being returned to the crude r, and then may be dried in a tunnel dryer. Such crystals constitute S.P. grade of calcium lactate. The washed crystals, without drying, be used in the manufacture of the best grades of lactic acid.

**rades of Lactic Acid.** Commercial, edible, and water-white grades ctic acid are manufactured by the Sheffield By-Products Company.

*actic Acid of Commercial Grade.* This grade of lactic acid is pro- d from the crude calcium lactate liquor obtained after the first num treatment (Fig. 52). This liquor, which has a concentration of Bé, is pumped to a wooden tank. Decolorizing carbon and chemical ts for precipitating iron and heavy metals are added. The mix is e slightly acid with an electrolytic grade of sulfuric acid and pumped ugh a rubber pipe to the upper tank of a vacuum filter made of stoneware. Calcium sulfate and other precipitated matter are rem The precipitates are washed while on the filter. The filter cake i carded, the filtrate and washings constituting a crude lactic acid sol of approximately 22 per cent strength.

The crude lactic acid may be stored in a wooden tank or further pu at once. The next step in the purification process is to concentrat crude acid to 22°Bé (50 to 60 per cent lactic acid) in a stainless- vacuum evaporator. The concentrated acid is conveyed to the upper of a vacuum filter, where Norit is added, together with sufficient lim sulfuric acid to produce exact combination of the calcium and sul Chemical agents may be added to precipitate any heavy metals present. The mix is filtered and the filter cake washed and dispose The filtered lactic acid is pumped to glass-lined tanks and adjuste the desired concentration. Lactic acids of 50 and 44 per cent con trations are in popular demand. The acid is placed in wooden barrels distribution.

*Lactic Acid of Edible Grade.* The starting material for the manu ture of the edible grade of lactic acid is the washed, solid, crude calc lactate that is obtained from the centrifuge after the first crystalliza (Fig. 52). This crude calcium lactate is added to a small amount of water in a wooden tank to form a solution. Sufficient sulfuric aci added to combine all the calcium and sulfate as calcium sulfate. Fin decolorizing carbon is added, and the mix is agitated thoroughly. precipitates are removed as a sludge by vacuum filtration. The lactic a is stored in wooden tanks and eventually concentrated to 50 to 60 cent strength in stainless-steel evaporating pans.

The purification treatment is repeated. Calcium and sulfate are exac balanced, and a decolorizing carbon and a reagent to precipitate hea metals are added. The mix is vacuum filtered, and the cake is wash In glass-lined tanks, the lactic acid of approximately 18°Bé is dilu to 50 or 44 per cent strength and placed in wooden barrels. Such aci water clear.

*Water-white Lactic Acid.* This grade is of very high purity and used by the chemical industry. Although either dry or wet U.S.P. calci lactate may be used for its manufacture, the wet product is most genera used, since the necessity for drying is eliminated.

The purification process is similar to that employed in the making edible-grade lactic acid. The calcium is exactly balanced with sulfa decolorizing carbon, etc., are added, and the mix is vacuum-filtered. acid from the vacuum evaporating pan is of 18°Bé strength or conta about 65 per cent of lactic acid.

This acid must be entirely freed of calcium. An excess of sulfuric a is used to precipitate the last traces of this substance. The filtered a should give no precipitate when tested with ammonium oxalate.

Barium hydroxide is added to remove the excess of sulfuric ac although a very slight excess of sulfuric acid is desired in view of the f that the water used for diluting the lactic acid may contain a sli amount of calcium. Thus the very slight excess of sulfuric acid will b he calcium added by the dilution water. A final filtration removes ecipitates of barium sulfate and calcium sulfate. Should distilled be used for diluting the water-white acid to its final strength, dilu- nay be made after the final filtration.

lactic acid is placed in barrels for marketing.

## LACTIC ACID FROM CHEESE WHEY

production of calcium lactate and lactic acid from albumin-free containing added nutrients, has been described by Campbell ).

starter is made from a culture of *Lactobacillus bulgaricus* C.C. 9224) by progressively increasing the volume of the mash as s: 20 ml., 200 ml., 2 liters, 5 gal., 50 gal., 500 gal., and 5,000 gal. nash for the first three stages contains albumin-free whey, 2 per cent n acetate, and 1 per cent corn-steep liquor and is sterilized. The two stages may be accomplished in glass-lined or stainless-steel , and the 500-gal. stage in a wooden tank. The medium (sterilized) ese three stages is the same as that for the first three stages except t contains 4 per cent $CaCO_3$ instead of 2 per cent sodium acetate. anks are fitted with mechanical agitators. The mash in the 5,000-gal. contains albumin-free whey and 0.5 or 0.65 per cent corn-steep r and is mechanically agitated. The pH may be maintained by the l addition of 4 per cent $CaCO_3$ or by the intermittent addition of of lime to keep it between 5 and 6.

e corn-steep liquor may be sterilized separately with the $CaCO_3$ in tion of the whey. Pipe lines and tanks must be cleaned and steamed lly before use.

fermentation is carried out at 110°F., the temperature in the tanks maintained by circulating steam or cold water through coils con- ed of bronze or brass and located on the interior. A yield of 85 to r cent lactic acid, based on the weight of lactose used, may be ned in 24 hr.

the end of the fermentation, the pH is elevated to about 12 with a calcium lime, the temperature is raised to the boiling point, and the ial is simmered gently for about 20 min. The precipitated calcium hate is filtered out, and the pH is brought to 7 with lactic acid. product may be recovered as lactic acid by using sulfuric acid or as um lactate by concentrating the solution, crystallizing the calcium te, washing the lactate, and drying it. The product obtained by dry- at a temperature of less than 212°F. is the pentahydrate; that ned by drying at 240°F., or above, is anhydrous.

## TIC ACID PRODUCTION BY CONTINUOUS FERMENTATION

procedure for producing lactic acid from the lactose of sweet whey semiplant basis by continuous fermentation was worked out by tier and Rogers (1931). The main equipment used in this procedure included a storage tank for whey; a lime reservoir and f device; an insulated and covered fermentation tank with faciliti maintaining a constant temperature of 43° ± 0.1°C. and for agitatin medium at a slow rate; a storage tank to receive the overflow from th mentation tank, which should have a capacity for 25 per cent o latter tank; and a coagulation tank, which should have 50 to 100 per of the operating capacity of the fermentation tank and which is conn by way of a filter press or centrifuge to an evaporator.

For operation, the fermentation tank is filled with sweet whey temperature of 43°C. The mash is inoculated with a culture of l bacilli, for example, *Lactobacillus bulgaricus* or *L. casei,* and somet with a yeast, which by reason of its associated growth accelerates the mentation. The inoculated medium is agitated and maintained at 4 (as closely as possible) for the duration of the fermentation.

After the pH of the mash has dropped to 5, usually after approxima 12 hr., lime is fed into it to maintain its pH between 5 and 5.8, a ra favorable to the lactic acid bacteria but inhibitory to the developmen contaminating organisms under the conditions of the fermentation.

At the end of 24 hr., and thereafter every 12 hr., the lactose conten the whey is ascertained. When the amount of lactose has become dir ished to less than 1 per cent, usually 48 to 72 hr. after the mash has b inoculated, whey, which may be pretreated with lime to inhibit the de opment of bacteria, is introduced into the fermentation tank from whey storage tank. The whey is introduced at such a rate that volume fed during 24 hr. equals the volume of the fermentation ta The rate is then adjusted for the most efficient operation. In this c nection, the lactose content of the overflowing whey should be de mined.

The fermented whey is boiled until the proteins are entirely coa lated. If lactic acid is desired, rather than calcium lactate, sulfuric a is added to precipitate the calcium and liberate free lactic acid. coagulated protein and calcium sulfate are then separated by filtrat from the lactic acid, which is concentrated in evaporators to the desi concentration.

## LACTIC ACID FROM CORN SUGAR

The commercial production of lactic acid from corn sugar has b described by Inskeep, Taylor, and Breitzke (1956). Reference is m to the flow sheet (Fig. 53) in this connection.

The medium used in the culture tanks and fermentors contained 15 cent corn sugar, 0.375 per cent malt sprouts, 0.25 per cent diammoni phosphate, 74.375 per cent water, and 10 per cent $CaCO_3$ as a buffer maintain the pH in the desired range of 5.8 to 6.

The culture tanks were charged with 375 gal. of mash. The ferment of 6,600-gal. and 30,000-gal. sizes, were filled to volumes of 4,600 gal. a 24,000 gal., respectively. The culture tanks and fermentors were p vided with mechanical agitators to keep the contents mixed. The te

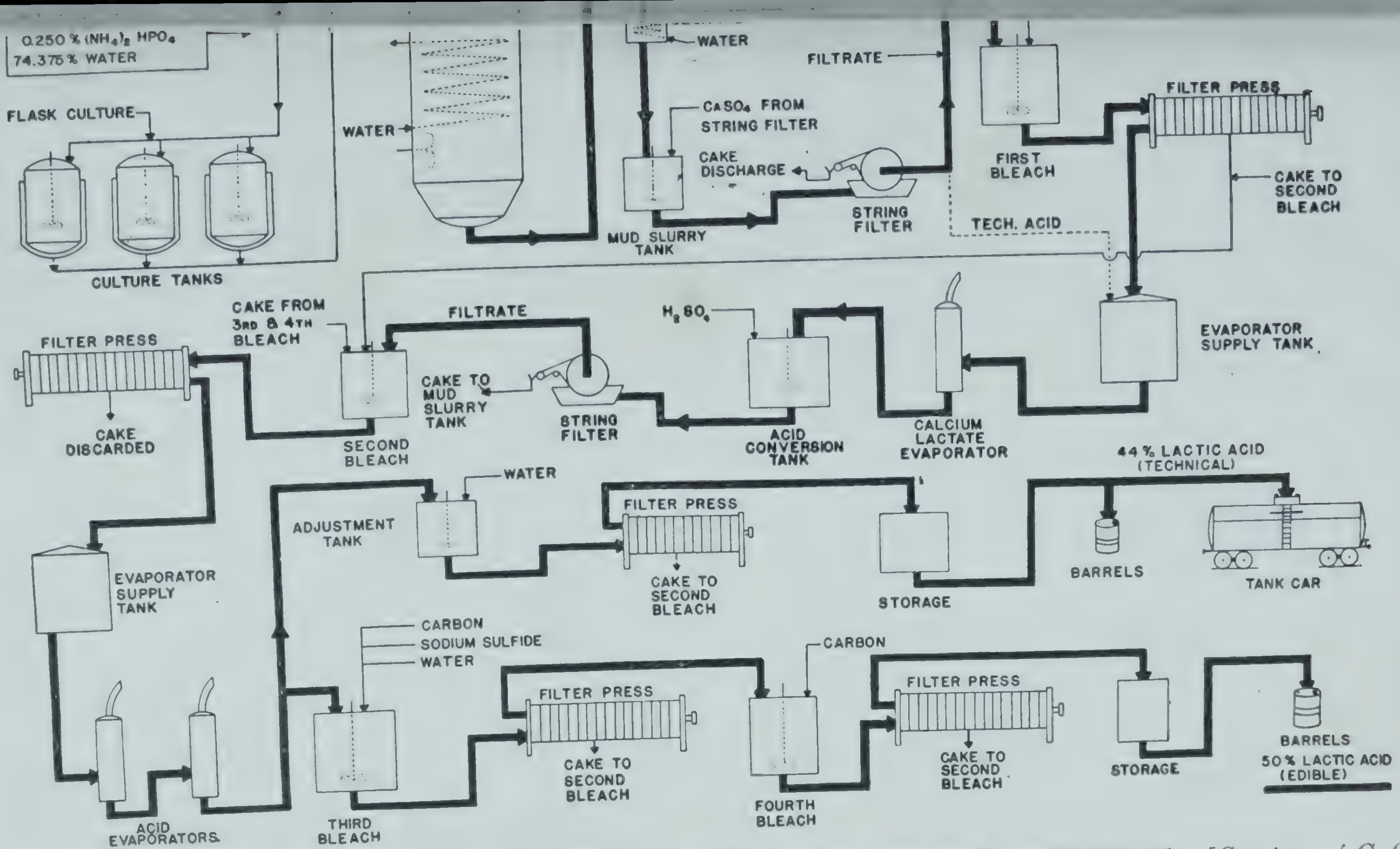

0.250 % $(NH_4)_2$ $HPO_4$
74.375 % WATER
FLASK CULTURE
CULTURE TANKS
WATER
MUD SLURRY TANK
WATER
$CaSO_4$ FROM STRING FILTER
CAKE DISCHARGE
STRING FILTER
FILTRATE
FIRST BLEACH
TECH. ACID
FILTER PRESS
CAKE TO SECOND BLEACH
EVAPORATOR SUPPLY TANK
CALCIUM LACTATE EVAPORATOR
$H_2SO_4$
ACID CONVERSION TANK
STRING FILTER
CAKE TO MUD SLURRY TANK
FILTRATE
CAKE FROM 3RD & 4TH BLEACH
SECOND BLEACH
FILTER PRESS
CAKE DISCARDED
EVAPORATOR SUPPLY TANK
ACID EVAPORATORS
ADJUSTMENT TANK
WATER
FILTER PRESS
CAKE TO SECOND BLEACH
STORAGE
44 % LACTIC ACID (TECHNICAL)
BARRELS
TANK CAR
CARBON
SODIUM SULFIDE
WATER
THIRD BLEACH
FILTER PRESS
CAKE TO SECOND BLEACH
CARBON
FOURTH BLEACH
FILTER PRESS
CAKE TO SECOND BLEACH
STORAGE
BARRELS
50 % LACTIC ACID (EDIBLE)

FIG. 53. Flow sheet for the production of lactic acid at Hammond, Ind., plant of American Maize-Products Co. [*Courtesy of G. C. Inskeep, G. G. Taylor, and W. C. Breitzke, Ind. Eng. Chem.*, 44: 1955 (1956). *Copyright 1956 by the American Chemical Society and reprinted by permission of the copyright owner.*]

perature was maintained at 120°F. (48.9°C.) by circulating water thr the stainless-steel jackets of the culture tanks and the stainless- coils of the fermentors.

The inoculum, *Lactobacillus delbrueckii,* was developed from the ture tube to a 500-ml. flask, to a 6-liter flask containing 3 liter medium, and to a culture tank. The contents of one culture tank used to seed a 6,600-gal. fermentor; those of three culture tank 30,000-gal. fermentor.

The course of the fermentation was followed daily by checking reducing sugar content of the medium and the pH. Fermentation considered to be complete when the reducing sugar content had droppe 0.10 per cent. The normal duration of the fermentation was 4 to 6 (refer to Fig. 54).

At the end of the fermentation, the mash was heated to 18 (82.2°C.) to destroy the bacteria.

Lactic acid, or calcium lactate, may be recovered as end product this fermentation, depending on the method of treatment of the fer tation product. The procedure used in recovering lactic acid is sh in Fig. 53; that used in recovering calcium lactate, in Fig. 55. The in ested reader is referred to the excellent article by Inskeep et al. (1 for additional details concerning recovery, purification, and p equipment.

## LACTIC ACID FROM BLACKSTRAP MOLASSES

A process for producing lactic acid and methyl lactate from blacks molasses has been described by Needle and Aries (1949).

A mash containing the molasses, chalk or limestone, and nutrient inoculated with a suitable culture of *Lactobacillus.* Fermentatio carried out at 50°C. until the sugar has been utilized, which usually t 5 to 7 days.

The mash contains the following ingredients: 20,000 lb. blacks molasses, 5,500 lb. chalk or whiting, 100 lb. diammonium phosph 400 lb. malt sprouts, and sufficient water to provide a mash of 10,000

The inoculum is built up through the following stages: 50-ml. cul tube, 500-ml. culture flask, 2.5-gal. culture flask, 50-gal. tank (in pla and 500-gal. tank (in plant). The incubation time for each stage, ex the last, is 24 hr.

Details for processing a plastic and U.S.P. grades of lactic acid methyl lactate, as well as a cost analysis for methyl lactate, are prese by the authors.

## LACTIC ACID FROM POTATOES

A method for producing lactic acid from potatoes has been descri by Cordon and associates (1950).

Ground whole potatoes were mixed with an equal weight of wate produce a mash which would yield 6 to 7 per cent sugars after saccha

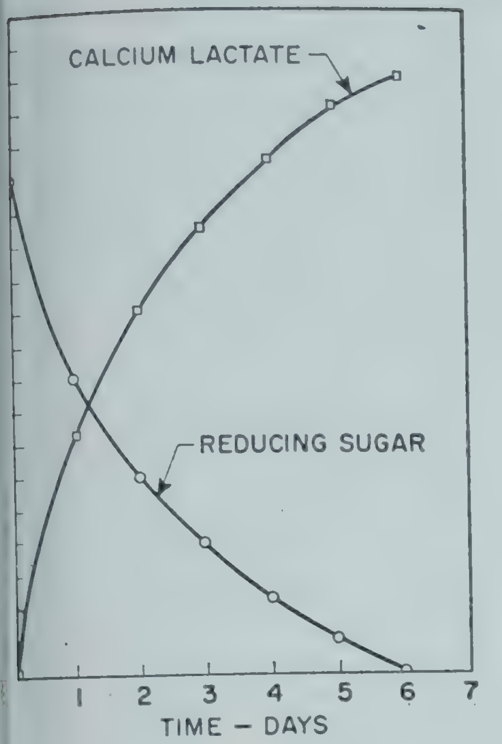
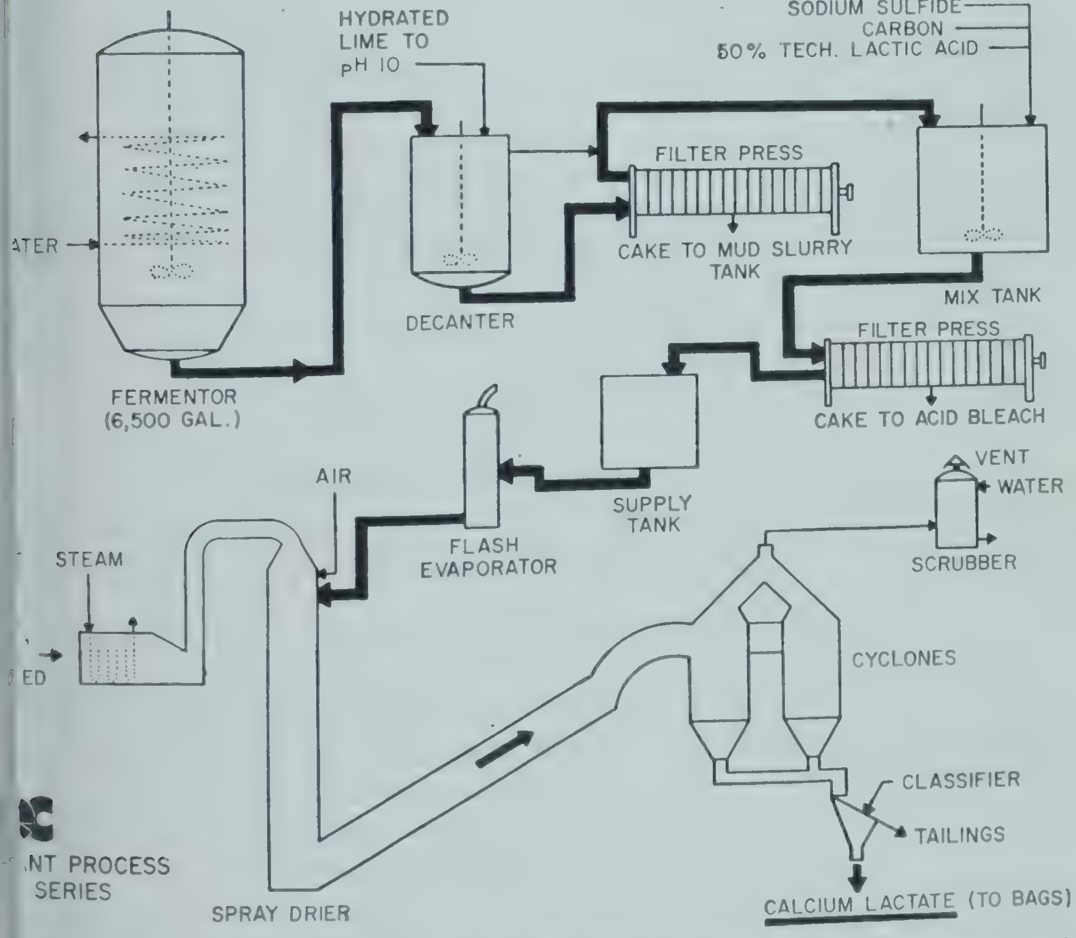


FIG. 54. Production of calcium lactate and decrease in reducing sugar during fermentation. [*Courtesy of G. C. Inskeep, G. G. Taylor, and W. C. Breitzke, Ind. Eng. Chem.,* **44**: 1955 (1956). *Copyright* 1956 *by the American Chemical Society and reprinted by permission of the copyright owner.*]


HYDRATED
LIME TO
pH 10
SODIUM SULFIDE
CARBON
50% TECH. LACTIC ACID
FILTER PRESS
CAKE TO MUD SLURRY
TANK
ATER
DECANTER
MIX TANK
FILTER PRESS
FERMENTOR
(6,500 GAL.)
CAKE TO ACID BLEACH
VENT
WATER
AIR
SUPPLY
TANK
STEAM
FLASH
EVAPORATOR
SCRUBBER
CYCLONES
ED
CLASSIFIER
TAILINGS
NT PROCESS
SERIES
SPRAY DRIER
CALCIUM LACTATE (TO BAGS)

55. Flow sheet for the production of calcium lactate at the Hammond, Ind., t of American Maize-Products Co. [*Courtesy of G. C. Inskeep, G. G. Taylor, and* *C. Breitzke, Ind. Eng. Chem.,* **44**: 1955 (1956). *Copyright* 1956 *by the American* *mical Society and reprinted by permission of the copyright owner.*]

cation. In order to help thin the mash, 0.5 to 1 per cent of fungal am liquor was added. The mixture was pasted at 100°C., cooked for 1 h 130°C., or for 2 hr. at 120°C., and cooled to the saccharification temp ture. The mash was saccharified for 1.5 hr. at 45°C. with 5 per cent liquid culture of *Aspergillus niger* NRRL 330. The mash, thus prep and containing an excess of $CaCO_3$, was inoculated with 10 per cen volume of a culture of a lactobacillus (16 to 24 hr. old) in a po medium. Reference to Table 94 will indicate that, although three diffe

Table 94. Yields of Lactic Acid Obtained with Different Species of Lactobacil

(Fermentation time, 112 hr.)

| Experiment no. | Culture | Maltase units | Fermentation temperature, °C. | Yields of lactic acid, % | | |
|---|---|---|---|---|---|---|
| | | | | Based on organic material | Based on original carbohydrate † | Based suga consu |
| 11 | *Lactobacillus delbrueckii* NRRL B-445 | 61 | 45 | 64.5 | 79.4 | 93.8 |
| 14 | *Lactobacillus* ‡ | 48 | 45 | 52.9 | 61.9 | 76.0 |
| 15 | *L. pentosus* 124–2 § | 75 | 30 | 77.1 | 91.2 | 95.2 |
| 16 | | 62 | 30 | 68.9 | 86.3 | 91.2 |
| 21 | | 70 | 30 | 70.4 | 84.4 | 87.9 |

* Courtesy of T. C. Cordon et al., *Ind. Eng. Chem.*, **42**: 1833 (1950). Copyr 1950 by the American Chemical Society and reprinted by permission of the c right owner.

† On moisture- and ash-free basis.

‡ Aerobic culture obtained from industrial concern; slow aeration provided du fermentation.

§ Obtained from W. H. Peterson, University of Wisconsin.

organisms were used, the best results were obtained with *Lactobaci pentosus* 124–2, at a temperature of 30°C. for fermentation. Yields 80 to 90 per cent were obtained in about 5 days, based on the origi carbohydrate.

The lactic acid was separated and recovered from the fermented m by the methanol vapor method of Filachione and Fisher (1946). mash was acidified to pH 1.9 to 2 with concentrated sulfuric acid digested for 2 hr. at 100°C. The digest was filtered and the filtrate c centrated by evaporation to 25 to 50 per cent of the original volu Methanol vapors were passed through the crude lactic acid at 100 The methyl lactate formed was volatilized in a stream of methanol va The mixture of lactate, methanol, and water was condensed. The c densate was heated to hydrolyze the methyl lactate and distill methanol. Purified lactic acid remained.

## THE PRODUCTION OF WHITE CALCIUM LACTATE

process (patented) for producing a white calcium lactate has been oped by Daly, Walsh, and Needle. The special features of this ss are the use of a nondenatured milk as the nutrient and the rapid ig of the calcium lactate produced.

iole milk, buttermilk, or skim milk, in liquid or in dried form, may sed as the nutrient, but the protein must not be denatured. Non-tured milk favors a rapid fermentation, thus avoiding the formation desirable colors or end products, and imparts no color to the medium. e rapid drying of calcium lactate prevents caramelization and other changes.

lcium lactate may be produced according to the following example. ical mash may contain the following ingredients:

| | Lb. |
|---|---|
| Sugar (dextrose) | 6,600 |
| Milk powder | 150 |
| Diammonium acid phosphate | 50 |
| Calcium carbonate | 4,600 |
| Water to 7,000 gal. | |

ie foregoing mash is inoculated with 300 gal. of a 24-hr. culture of *obacillus delbrueckii* and incubated at a temperature of 49°C. °F.) for 5 to 7 days, at the end of which time the total reducing rs are usually less than 0.1 per cent. The use of the high temperature rs *L. delbrueckii* and inhibits the development of many other types icroorganisms.

e fermented mash is heated to 82°C. (180°F.) and held at that erature for sufficient time to destroy the microorganisms present. igh lime is then added (100 to 200 lb.) to bring the pH of the mash pproximately 11. The mash is filtered hot, proteins and insoluble ounds being removed. The filtrate is adjusted to a pH of 6 to 7 with acid, for this pH range favors the volatilization of undesired iic acids and tends to prevent the formation of undesired color during ubsequent treatment. Using a vegetable carbon, the calcium lactate r is decolorized to yield a solution of water-white color. The solution ncentrated quickly to 20 to 21°Bé by exposing a shallow layer to a heating surface. This partially concentrated liquor is next spray-l in a chamber into which gases enter at a temperature of approxi-ely 232°C. (450°F.) and leave at a lower temperature. (Other types rying may be substituted, if the drying is effected rapidly to prevent loration.) The resultant powder, maintained at a low temperature der to avoid discoloration, is conveyed to a cyclone, where it may be rated into relatively fine and coarse particles.

ie product obtained by the foregoing process is noncrystalline, glass-and homogeneous, with a calcium lactate content, based on the total s, of 98 to 99 per cent. The moisture content is 5 to 6 per cent; the ein, usually about 0.5 per cent.

If lactic acid is desired instead of calcium lactate, the filtrate fr the lime treatment is decolorized with vegetable carbon and filtered. measured quantity of sulfuric acid is then added to the filtrate, and t precipitate of calcium sulfate is removed by filtration. The resulta filtrate is decolorized with vegetable carbon and then concentrated to t desired strength by evaporation.

## THE PRODUCTION OF DEXTROLACTIC ACID

Tatum and Peterson (1935) have described a method for produci L(+)-lactic acid on a small scale. Batches containing 18 liters of medium consisting of 3 per cent cerelose and 3 per cent malt sprouts we sterilized in 20-liter Pyrex bottles. The medium was inoculated with pure culture of an organism that produced L(+)-lactic acid. The inoc lated medium was incubated at the temperature most favorable for t organism used, 30 or 37°C. After the fermentation had been in progre for 24 hr., an excess of sterile calcium carbonate was added. Each bott was shaken frequently during the 6 to 10 days of incubation in order neutralize the acid as it was formed. Samples of the acid were convert to zinc lactate for analyses. Table 95 gives data for some of the ferment tions carried out by Tatum and Peterson.

TABLE 95. LACTIC ACID PRODUCTION BY VARIOUS ORGANISMS *

| Organism | Temperature of incubation, °C. | Lactic acid formed per 540 g. of glucose, g. | Glucose converted to lactic acid, per cent | Analysis of zinc lactate | | Form lactic acid |
|---|---|---|---|---|---|---|
| | | | | Water of crystallization, per cent | Specific rotation,† $[\alpha]_D^{20}$ | |
| *Streptococcus lactis*, R. . . . | 30 | 495 | 91 | 12.52 | −8.65 | Dext |
| *Strep. lactis*, R. . . . . . . . . . | 30 | 525 | 97 | 13.23 | −8.65 | Dext |
| *Lactobacillus casei* . . . . . . . | 30 | 505 | 93 | 12.80 | −8.22 | Dextr |
| *L. delbrueckii*, 3. . . . . . . . . . | 37 | 530 | 98 | 13.00 | −8.22 | Dextr |
| *L. delbrueckii*, 3. . . . . . . . . . | 37 | 520 | 96 | 12.90 | −8.18 | Dextr |
| *L. delbrueckii*, 3. . . . . . . . . . | 37 | 500 | 92 | 12.86 | −8.60 | Dextr |

* E. L. Tatum and W. H. Peterson, *Ind. Eng. Chem.*, 27: 1493 (1935). Copyrigl 1935 by the American Chemical Society and reprinted by permission of the copyrigl owner.

† 4 per cent concentration.

## LACTIC ACID FROM SULFITE WASTE LIQUOR

The production of lactic acid from sulfite waste liquor has been thor oughly studied by Leonard, Peterson, and Johnson (1948). They foun that the best method for preparing the sulfite waste liquor for fermenta tion was as follows: The liquor was steam-stripped to remove the sulfu dioxide. During this treatment the pH was reduced to about 4. Next th

or was treated with calcium hydroxide at 35°C. in such manner that
quired about 10 min. to raise the pH from 4 to 8.5. The mixture was
at pH 8.5, the region of sulfite precipitation, for 20 to 30 min. and
filtered. Carbon dioxide was used to reduce the pH of the filtered
um to 7 or lower.

*ctobacillus pentosus* 124–2 was found to be superior to other bac-
for producing lactic acid from sulfite waste liquors. It was prepared
noculation purposes by repeated transfers at 8-hr. intervals in a
um containing 3 per cent of malt sprouts and 3 per cent of glucose.

e inoculum was prepared by growing *L. pentosus* for 8 hr. at 30°C.
mash containing 8 per cent of malt sprouts and 5 per cent of molasses.
mash was prepared by steeping the malt sprouts (for example, 16 to
er cent concentrations at 45°C. for 8 to 12 hr.), adding dilute molas-
eating to 100°C. for 10 min., and cooling to 30°C.

onard and his associates found that nutrients could not be sterilized
e presence of sulfite waste liquor, for the lignin substances precipi-
l and bound some of the nutritive materials with the result that lower
ls of lactic acid were obtained. The nutrients for the sulfite waste
or were, accordingly, supplied with the inoculum.

e sulfite liquor, prepared as described above, was inoculated with
er cent by volume of the 8-hr. inoculum. The pH, which was initially
t 6.5, usually fell to about 5.6 in 2 to 3 hr. It was maintained at
t this level by the automatic addition of a slurry of slaked lime or
um carbonate. Incubations were carried out for 40 to 48 hr. at 30°C.

e lactic acid was recovered from the fermented liquor in the follow-
manner: The malt sprouts were removed by screening. The liquor
then concentrated to approximately 40 per cent of solids (30 per cent
initial volume). The pH of the concentrate was reduced to 2 by the
tion of sulfuric acid and the precipitate of calcium sulfate formed
ved by filtration or centrifuging. The concentrated residue was
cted with a solvent (amyl alcohols and isophorone were good) at
. This extract was washed with water to remove the acids (lactic
acetic). The aqueous solution of the acids was concentrated and the
c acid separated out by distillation. The final product contained
r cent of lactic acid, 6 per cent of impurities, and 4 per cent of water.

onard and his coworkers estimated that a mill that produced 100
of pulp daily could produce 9,000,000 lb. of lactic acid annually on
asis of a 300-day year and of the production of 300 lb. of lactic acid
the sulfite waste liquors corresponding to 1 ton of pulp. Actually
t 285 lb. of lactic acid and 75 lb. of acetic acid may be recovered
2,000 gal. of sulfite waste liquor, which corresponds to 1 ton of pulp,
5 per cent recovery efficiency.

## L(+)-LACTIC ACID FROM JERUSALEM ARTICHOKES

e production of L(+)-lactic acid from Jerusalem artichokes has been
ied by Andersen and Greaves (1942). The tubers were washed and
nd. Two parts of water were added. The mixture was hydrolyzed

by adjusting the pH to 2 with sulfuric acid and heating for 1 hr. at 95 Complete hydrolysis occurred under these conditions with no apprecia destruction of the reducing sugars. The hydrolyzate was neutralized w calcium carbonate and filter-pressed through a cloth.

Nutrients, calcium carbonate, and water were added to the dilu hydrolyzate in the following optimum concentrations:

| | |
|---|---|
| Artichoke hydrolyzate (diluted 1 + 2) | 500 ml. |
| $(NH_4)_2SO_4$ | 3.0 g. |
| $KH_2PO_4$ | 0.5 g. |
| Glucose or levulose | 110.0 g. |
| $CaCO_3$ | 75.0 g. |
| Water | 430 ml. |

The amount of sugar added was sufficient to produce an over-all su concentration of about 14 g. per 100 ml. The quality of the final pro uct could be improved by adding glucose or levulose to the dilut hydrolyzate.

The foregoing medium, dispensed in 200-ml. portions in 500-ml. Erle meyer flasks, or in 2,000-ml. portions in 3-liter Fernbach flasks, w sterilized either by boiling or by autoclaving at a pressure of 15 lb. per in. for 15 min. After cooling to 47 to 50°C., it was inoculated with 5 10 per cent by volume of a 24-hr. culture of *Bacillus dextrolacticus* Ande sen and Werkman.

The fermentations were carried out at 47 to 50°C. with agitation a aeration. The latter was essential for rapid and complete fermentati of the sugar. Yields of 92 to 94 per cent of L(+)-lactic acid were obtain in 4 to 5 days from the 14 per cent nutrient sugar solution as describ above.

## ACCELERATION OF THE FERMENTATION

Pan, Peterson, and Johnson (1940) reported on a method for accelera ing the lactic acid fermentation of glucose or molasses by the use unheated malt sprouts as a nutrient for *Lactobacillus delbrueckii.* T increased speed of fermentation was due to a heat-labile growth fact contained in the malt sprouts.

The following example illustrates the method employed by Pan a his associates: 64 gal. (240 liters) of diluted molasses (12.6 per ce invert sugar) were mixed with 14.7 lb. (6.67 kg.) of malt sprouts a heated to 45°C. in an open copper tank of 90-gal. capacity, which w provided with heating coils. This mash was inoculated with 4 liters of 24-hr. culture of *L. delbrueckii,* which had been grown in a steam medium containing 3 per cent of malt sprouts and 10 per cent of molass Then 33 lb. of calcium carbonate was added. During the fermentati the mash was stirred occasionally and the temperature was maintain within the range of 44 to 46°C. The fermentation was complete in 21 h Data concerning this fermentation and one carried out with Puerto Rica blackstrap molasses, in which a mechanical agitator was used to aid eutralization and maintenance of a uniform temperature, are pre- d in Table 96.

TABLE 96. DATA OF LARGE-SCALE FERMENTATIONS *, †

| | Run 1 | Run 2 ‡ |
|---|---|---|
| Molasses used, lb. | 120.2 | 100.0 |
| Sugar content of molasses, per cent | 55.9 | 59.5 |
| Malt sprouts, lb. | 14.7 | 14.5 |
| $CaCO_3$ added, lb. | 33.0 | 32.5 |
| Total volume, gal. | 63.8 | 63.0 |
| Duration of fermentation, hr. | 21 | 16 |
| Initial sugar concentration, g./100 ml. | 12.6 | 11.32 |
| Final sugar concentration, g./100 ml. | 1.10 | 1.46 |
| Fermentation, per cent | 91.3 | 87.0 |
| Lactic acid, g./100 ml. | 11.0 | 9.35 |
| Yield, per cent sugar fermented | 95.7 | 95.0 |
| Yield, per cent sugar in molasses | 87.3 | 82.6 |
| Sp. rotations § of Ca lactate (4 per cent), $[\alpha]_D$ | +6.16 | +6.06 |
| Form of lactic acid | levo | levo |

C. Pan, W. H. Peterson, and M. J. Johnson. *Ind. Eng. Chem.*, 32: 709 (1940).
right 1940 by the American Chemical Society and reprinted by permission of
pyright owner.
*actobacillus delbrueckii* g used; 44 to 46°C.
erto Rican blackstrap molasses.
alue of $[\alpha]_D$ for calcium lactate is 6.13.

## THE PURIFICATION OF LACTIC ACID

the purification of lactic acid, the object is to remove all ingredients constitute impurities. These may include unfermented sugar, genous substances, coloring matter, calcium or sodium sulfate, and salts.

veral methods may be used in purifying lactic acid. Smith and Cla- (1939), of the Bureau of Dairy Industry, U.S. Department of Agri- re, have briefly discussed six methods for accomplishing purification. method is purification by the process of recrystallizing calcium lac- followed by treatment with sulfuric acid to liberate lactic acid. process was described in detail in a foregoing section that dealt the production of lactic acid from whey. Olive and Burton have ribed this process.

second method consists in converting calcium lactate to zinc lactate, h crystallizes more readily than other lactates. The zinc lactate is ied by repeated crystallizations. By adding hydrogen sulfide to zinc te, lactic acid is liberated and zinc sulfide precipitated. Animal char- is added to decolorize the mass, which is then filtered. The filtrate, h contains the lactic acid, is concentrated by evaporation in vacuo. another method, esters of lactic acid are prepared, purified, and equently hydrolyzed to liberate lactic acid in a pure form. Methanol

($CH_3OH$) is added to the lactate in the proportion of 10 to 20 mo
methanol to 1 mole of calcium lactate or 2 moles of sodium lactate, w
preferably, should be free from moisture. Any undissolved precip
are removed by filtration. Sulfuric acid is added, which accomp
two objectives, liberation of lactic acid and catalysis of the subse
esterification. In order to esterify the lactic acid, the mixture is h
for 4 to 8 hr. at a refluxing temperature. The precipitated subst
are removed by filtration, and the excess methanol by distillati
atmospheric pressure. Using a low temperature, the water and mo
the methyl lactate are distilled under vacuum. After diluting the d
late in the proportion of 2 to 3 parts of distilled water to 1 part of disti
it is slowly fractionated in a corrosion-resistant column at atmosp
pressure. After hydrolysis of the methyl lactate, the methanol is re
ered and the liberated lactic acid concentrated by evaporation in va
Smith and Claborn state that chemically pure lactic acid may be prep
most efficiently and economically by this method.

Lactic acid may be partially purified in another process by ge
oxidizing the crude liquor, which contains the lactates or lactic
Various oxidizing agents have been used for this purpose: sodiu
calcium hypochlorite, potassium permanganate, potassium chron
nitric acid, hydrogen peroxide, chlorine gas, and ozone gas.

Lactic acid may be extracted from its water solution by the us
various solvents, one of which is isopropyl ether. This process is sai
be expensive and hazardous, on account of the inflammability of the e
and possibility of explosive peroxides being formed.

Fractional distillation has not been used successfully on a comme
scale.

A method for purifying lactic acid and preparing a methyl lac
directly from the crude aqueous acid has been described by Filach
and Fisher (1946). The vapor of methanol is passed through the aqu
lactic acid. The effluent vapors, which are a mixture of methanol, wa
and methyl lactate, are condensed. The condensate may be distille
order to recover the methyl lactate, or it may be hydrolyzed to ob
purified lactic acid. Through a similar use of other alcohols, other es
of lactic acid may be obtained.

## CONSTRUCTION MATERIALS

Lactic acid is highly corrosive and therefore presents special probl
in relation to its production, processing, and handling. These probl
have been discussed by Woodward (1950), Filachione (1952), Insk
et al. (1956), and others.

Two of the materials of construction which are most resistant to la
acid—silver and tantalum—are too expensive to use, except for spe
purposes, such as in the coating of valves used in filters. A group
materials which is fairly resistant includes nickel, Inconel, and low-
alloys which contain high percentages of nickel and chromium (stain

. Materials of relatively poor resistance and of limited life include
hrome steel, copper, copper alloys, and monel metal.
inless steel and wood are two of the most practicable materials for
connection with lactic acid production and processing. Wood tends
out when used for the storage of concentrated lactic acid.

## CONTAINERS USED IN DISTRIBUTION

eous solutions of lactic acid are sold and distributed in wooden or
in-lined barrels of oak, cypress, or pitch pine or in lined tank cars.
igher grades of the acid are sold in glass-lined cars or carboys
dward, 1950; Filachione, 1952).

## USES OF LACTIC ACID

tic acid has many uses. These include uses in connection with foods,
ntations, pharmaceuticals, and the chemical industries. As an
lant, the edible grade of lactic acid is used in confectionery, extracts,
juices and essences, lemonades, pickles, sirups, and other products.
c acid may be used in the curing of meat and in canned vegetable
fish products. It acts as a preservative and prevents putrefactive
ges from taking place in sauerkraut and pickles. It is used to acidu-
worts in the manufacture of beer, to adjust the pH of the brine in
anufacture of pickled green olives, and to inhibit the development
tyric acid bacteria in the manufacture of yeast. It is used in making
bets and effervescent beverages.

the chemical industries, lactic acid is used in the dyeing of silks
ther textile goods, as a mordant in the printing of woolens, in the
g and plumping of leathers, in the deliming of hides, in vegetable
ng, and as a flux for soft solders. The water-white grade is used in
lastic industry.

e lactates also have important uses. Calcium lactate is used in
g powders, in bread, in the pharmaceutical trade, and for other
ses. Iron lactate is used in pharmaceutical manufacture. Sodium
te is employed to aid in the retention of moisture by such products
bacco. Copper lactate is a very important agent in a new process
e electrolytic deposition of metals.

es have been found for derivatives of lactic acid (refer to Table 97).
rs of lactic acid, which may be represented by the general formula
$\cdot\mathrm{CHOR}\cdot\mathrm{COOH}$ and which are insoluble in water, may be used as
nts, plasticizers, and modifiers in the manufacture of inks, plastics,
lacquers. Esters of lactic acid, which have the general formula
$\cdot\mathrm{CHOH}\cdot\mathrm{COOR}$, may be used for many of the same purposes as the
s. The higher esters, for example, the butyl, amyl, and lauryl esters,
ss greater stability than the lower esters, are insoluble in water, and
herefore more desirable than the latter.

Table 97. Commercially Available Derivatives of Lactic Acid and U

| Derivative | Formula | Use |
|---|---|---|
| Antimony lactate .......... | $(CH_3CHOHCOO)_3Sb$ | In mordant dy |
| Calcium lactate ........... | $(CH_3CHOHCOO)_2Ca\cdot 5H_2O$ | Calcium therap |
| Copper lactate ............ | $(CH_3CHOHCOO)_2Cu\cdot 3H_2O$ | Electroplating b |
| Iron lactate .............. | $(CH_3CHOHCOO)_2Fe\cdot 3H_2O$ | Treatment of an |
| Sodium lactate ............ | $CH_3CHOHCOONa$ | Plasticizer, hum tant |
| Strontium lactate .......... | $(CH_3CHOHCOO)_2Sr\cdot 3H_2O$ | Pharmaceutical |
| *n*-Butyl lactate ........... | $CH_3CHOHCOO(CH_2)_3CH_3$ | Solvent in lacqu |
| Ethyl lactate ............. | $CH_3CHOHCOOCH_2CH_3$ | Solvent, lubrican |
| Methyl lactate ............ | $CH_3CHOHCOOCH_3$ | Intermediate |

## MECHANISM OF THE LACTIC ACID FERMENTATIONS

**Homofermentative Bacteria.** It has been suggested that the ini stages in the lactic acid fermentation may be similar to those of the et alcohol fermentation. Phosphates added to a mash accelerate the f mentation. The removal of coenzyme from lactic acid bacteria w retard or cause the fermentation to cease. *Lactobacillus delbrueckii* h the ability to convert hexosediphosphate to methylglyoxal, likewise convert methylglyoxal quantitatively to racemic lactic acid.

Pyruvic acid is an important intermediate in the formation of lac acid by homofermentative bacteria. It is believed to arise by a mec anism similar to that shown to exist in the ethanol fermentation yeast. The pyruvic acid formed by the lactic acid bacteria is acted up by a dehydrogenase (of which there appear to be at least three kin formed by bacteria) and converted to lactic acid.

The total changes may be expressed by the following equation:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Lactic acid}}{2CH_3\cdot CHOH\cdot COOH}$$

**Heterofermentative Bacteria.** Some of the first researches concer ing the mechanism of the formation of the final products by heter fermentative lactic acid bacteria were carried out by Gayon and Dubou (1901); by Fred, Peterson, and Davenport (1920); by Peterson and Fr (1920); and by others.

Nelson and Werkman (1935) carried out experiments with seve heterofermentative bacteria and obtained data upon which they ha based a scheme for the dissimilation of glucose. The medium used these men contained 2 per cent glucose, 1 per cent peptone, 0.3 per ce yeast extract (Difco), 0.6 per cent $K_2HPO_4$, and 0.6 per cent $KH_2P$ with a pH of 6.2. Of these constituents, (1) the glucose, (2) the pepto and yeast extract, and (3) the phosphates were sterilized separately f 20 min. under a steam pressure of 20 lb. The separately sterilized com ponents were combined just before inoculation. The mashes were inc bated under an atmosphere of oxygen-free nitrogen at 30°C. for a peri of 3 weeks before being analyzed.

The data of Table 98 illustrate the kinds and quantities of the produ

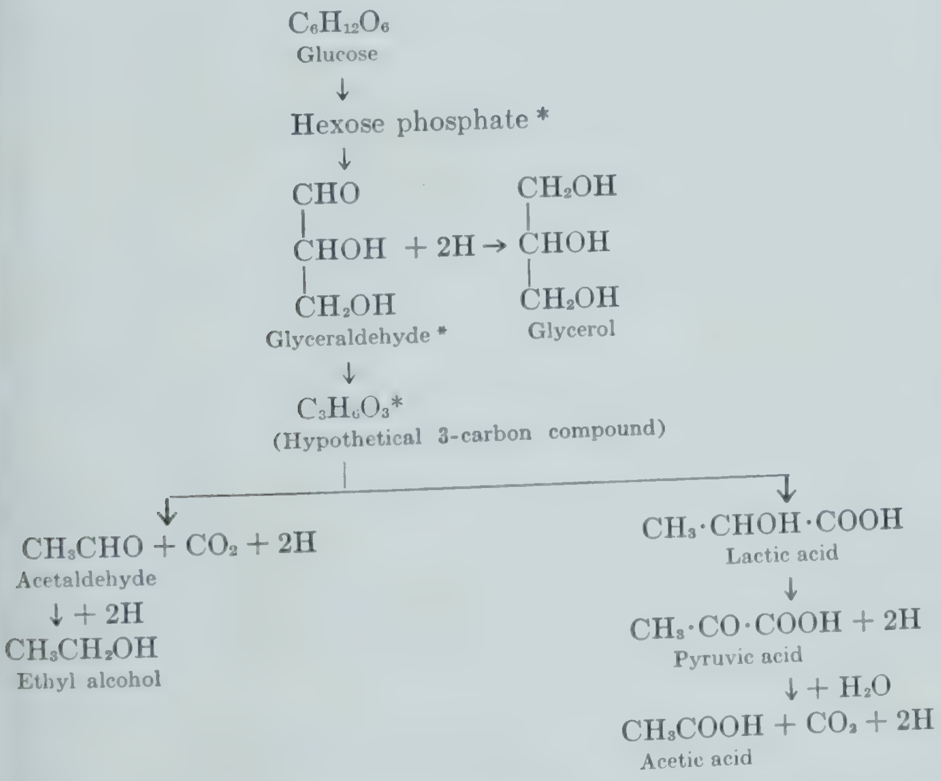
8. ANAEROBIC DISSIMILATION OF GLUCOSE BY *Lactobacillus acidophil-aerogenes* AND *L. lycopersici* *

| Products | *L. acidophil-aerogenes,* mM./liter | *L. lycopersici,* mM./liter | | | |
|---|---|---|---|---|---|
| | | I | II | III | IV |
| fermented | 88.9 | 80.0 | 92.1 | 102.2 | 112.2 |
| l alcohol | 39.5 | 63.0 | 69.6 | 74.1 | 78.9 |
| c acid | 10.2 | 12.1 | 16.5 | 15.5 | 19.5 |
| on dioxide | 43.9 | 64.3 | 80.0 | 81.0 | 95.1 |
| c acid | 108.2 | 65.2 | 67.6 | 83.1 | 89.7 |
| rol | 21.7 | 25.0 | 36.2 | 32.6 | 43.5 |
| nic acid | 0.8 | 3.8 | | | |
| tage of carbon recovered: | | | | | |
| succinic acid | 100.2 | 104.2 | | | |
| out succinic acid | 100.0 | 101.0 | 101.9 | 99.0 | 103.0 |
| on-reduction ratio | 0.873 | 0.851 | 0.912 | 0.884 | 0.943 |

. E. Nelson and C. H. Werkman, *Jour. Bact.*, 30: 547–557 (1935).

d by *L. acidophil-aerogenes* and *L. lycopersici* from glucose under the mental conditions just mentioned. (*L. acidophil-aerogenes* and *L. rsici* are listed as probable synonyms of *L. brevis* in the seventh edi- f "Bergey's Manual of Determinative Bacteriology.")

son and Werkman have suggested a tentative scheme for the dis- tion of glucose by heterofermentative bacteria. This scheme is below.

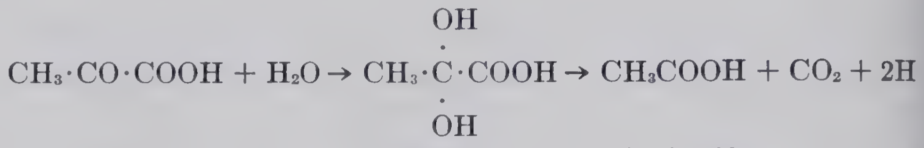

$C_6H_{12}O_6$
Glucose
Hexose phosphate *
CHO
CHOH + 2H →
$CH_2OH$
Glyceraldehyde *
$CH_2OH$
CHOH
$CH_2OH$
Glycerol
$C_3H_6O_3$*
(Hypothetical 3-carbon compound)
$CH_3CHO + CO_2 + 2H$
Acetaldehyde
+ 2H
$CH_3CH_2OH$
Ethyl alcohol
$CH_3 \cdot CHOH \cdot COOH$
Lactic acid
$CH_3 \cdot CO \cdot COOH + 2H$
Pyruvic acid
+ $H_2O$
$CH_3COOH + CO_2 + 2H$
Acetic acid

ese products have not been identified in the fermentation.

In the foregoing scheme, acetaldehyde and pyruvic acid are impo intermediates. When acetaldehyde, or acetylmethylcarbinol, is add a glucose fermentation, there is an increase in the quantities of a acid and and carbon dioxide, but a decrease in the quantities of eth lactic acid, and glycerol formed from glucose. Since both acetalde and acetylmethylcarbinol are hydrogen acceptors, they will com with the hydrogen acceptors, such as glyceraldehyde, which arise mally during the fermentation. The result will be a decrease in the q tities of the compounds that are usually formed by reduction from t intermediary hydrogen acceptors.

The acetaldehyde added to a fermenting medium is reduced to eth

Nelson and Werkman (1936) have shown that pyruvic acid may be mented by *L. lycopersici* with the production of equimolar amoun acetic, lactic, and carbonic acids:



$$\mathrm{CH_3\cdot CO\cdot COOH + H_2O \rightarrow CH_3\cdot\overset{OH}{\underset{OH}{C}}\cdot COOH \rightarrow CH_3COOH + CO_2 + 2H}$$

Pyruvic acid Hydrated pyruvic acid Acetic acid

$$\mathrm{CH_3\cdot CO\cdot COOH + 2H \rightarrow CH_3\cdot CHOH\cdot COOH}$$

Pyruvic acid Lactic acid

One molecule of pyruvic acid is hydrated to form 1 molecule each acetic acid and carbon dioxide (carbonic acid), while a second molec is reduced to a molecule of lactic acid.

DeMoss et al. (1951) and Gunsalis and Gibbs (1952) have shown t equimolar quantities of D(−)-lactic acid, ethanol, and carbon dioxide formed from glucose by *Leuconostoc mesenteroides* strain 39; that lactic acid arises from carbon atoms 4, 5, and 6 of the glucose molecu that the carbon of the methyl group of ethanol arises from carbon 2; t carbon dioxide arises from carbon 1; and that no aldolase and isomer appear to be present in this organism.

The reader is referred to the review article by Gunsalis, Horecker, a Wood (1955) for additional details on this subject.

## PUBLICATIONS OF THE U.S. DEPARTMENT OF AGRICULTUR

Personnel of the Eastern Utilization Research Branch, U.S. Depa ment of Agriculture, have carried out an immense amount of research lactic acid and its derivatives. *Report AIC* 294 of the Eastern Utilizati Research Branch, compiled by Fisher, Dixon, and Filachione (195 contains 1,488 references concerned with the manufacture, recovery a purification, uses, chemistry and reactions, and derived products of lac acid and its derivatives.

### References on the Lactic Acid Fermentation

Andersen, A. A., and J. E. Greaves: *d*-Lactic Acid Fermentation of Jerusalem A chokes, *Ind. Eng. Chem.*, **34**: 1522–1526 (1942).

and C. H. Werkman: Description of *d*-Lactic Acid Forming Organisms
the Genus Bacillus, *Iowa State Coll. Jour. Sci.*, **14**: 187–193 (1940).
son, C. G.: "An Introduction to Bacteriological Chemistry," 2d ed., The
illiams & Wilkins Company, Baltimore, 1946.
R. S., and H. C. Needle: Producing Lactic Acid, U.S. Patent 2,588,460, Mar. 11,
52.
eau, C.: *Jour. Pharmacol.*, **12**(3): 244–261 (1847).
o, V.: Ricerche sui fermenti latto-mannitici. II. L'Acido lattico nella fer-
ntazione mannitica del levulosio, *Ann. chim. appl.*, **26**: 24–30 (1936).
: Ricerche sui fermenti latto-mannitici. III. I prodotti della fermentazione
l glucosio, *Ann. chim. appl.*, **26**: 356–361 (1936).
: Ricerche sui fermenti latto-mannitici. IV. L'influenza della reazione del
ezzo sulla fermentazione del glucosio e del fruttosio, *Ann. chim. appl.*, **26**: 423–
7 (1936).
: *Enzymologia*, **5**: 52 (1938).
, L. V.: Byproducts of Milk, *Food Inds.*, **9**: 571, 634 (1937).
ell, L. A.: Production of Calcium Lactate and Lactic Acid from Cheese Whey,
*m. Dairy Ice Cream Jour.*, **32**(3): 29–31 (1953).
n, T. C., R. H. Treadway, M. D. Walsh, and M. F. Osborne: Lactic Acid from
otatoes, *Ind. Eng. Chem.*, **42**: 1833–1836 (1950).
ss, R. D., R. C. Bard, and I. C. Gunsalis: The Mechanism of the Heterolactic
ermentation: A New Route of Ethanol Formation, *Jour. Bact.*, **62**: 499–511
951).
ione, E. M.: "Encyclopedia of Chemical Technology," Interscience Publishers,
c., New York, 1952, Vol. 8, pp. 167–180.
, E. J. Costello, T. J. Dietz, and C. H. Fisher: Lactic Acid Derivatives as
asticizers Esters of Polymeric Lactic Acid, *U.S. Dept. Agr., Bur. Agr. and Ind.*
*hem., Mimeographed Circ. Ser., AIC* 295, 1951.
and C. H. Fisher: Purification of Lactic Acid. Production of Methyl Lactate
om Aqueous Solutions of Crude Acid, *Ind. Eng. Chem.*, **38**: 228–232 (1946).
, C. H., M. B. Dixon, and E. M. Filachione: Bibliography on Lactic Acid and
erivatives, *U.S. Dept. Agr., Bur. Agr. and Ind. Chem., Mimeographed Circ. Ser.,*
*IC* 294, February, 1951.
and E. M. Filachione: Properties and Reactions of Lactic Acid—A Review,
*S. Dept. Agr., Bur. Agr. and Ind. Chem., Mimeographed Cir. Ser., AIC* 279,
tober, 1950.
E. B., W. H. Peterson, and A. Davenport: *Jour. Biol. Chem.*, **42**: 175–189
920).
mann, T. E., M. Cotonio, and P. A. Shaffer: Determination of Lactic Acid,
*ur. Biol. Chem.*, **73**: 335–358 (1927).
t, J. F.: Lactic Acid, *Ind. Eng. Chem.*, **22**: 1153–1154 (1930).
is, I. C., and M. Gibbs: The Heterolactic Fermentation. II. Position of C$^{14}$
the Products of Glucose Dissimilation by *Leuconostoc mesenteroides*, *Jour.*
*ol. Chem.*, **194**: 871–875 (1952).
, B. L. Horecker, and W. A. Wood: Pathways of Carbohydrate Metabolism
Microorganisms, *Bact. Revs.*, **19**: 79–128 (1955). (This article contains 431
ferences.)
ner, B. W., and F. J. Babel: "Dairy Bacteriology," 4th ed., John Wiley & Sons,
c., New York, 1957.
ep, G. C., G. G. Taylor, and W. C. Breitzke: Lactic Acid from Corn Sugar, *Ind.*
*ng. Chem.*, **44**: 1955–1966 (1956).
iri, H., and K. Kitahara: On the Optical Properties of the Fermentation
ctic Acids. I. The Lactic Acid Bacteria Having the Special Nature of Caus-
g Fermentation Lactic Acids to Form Inactive, *Bull. Agr. Chem. Soc. Japan*,
: 997–1007 (1935).
and ———: II. The Manner of Lactic Acid Bacteria towards Inactive and
tive Lactic Acids Added to the Fermentation Liquids, *Bull. Agr. Chem. Soc.*
*pan*, **12**: 96–100 (1936).
and ———: Part III. The Action of the Inactive Lactic Acid-producing

Organisms upon the Optically Active Lactic Acids Added to the Ferment Liquids, *Bull. Agr. Chem. Soc. Japan,* **12**: 281–283 (1936).

——— and ———: Part IV. A New Enzyme, Racemiase, Which Reveals Racem tion of Lactic Acids, *Bull. Agr. Chem. Soc. Japan,* **12**: 844–852 (1936).

——— and ———: Part V. The Action of Acetone-Butyl Alcohol Producing Or isms upon Optically Active Lactic Acids, *Bull. Agr. Chem. Soc. Japan,* **12**: 1 1220 (1936).

——— and ———: Racemiase, an Enzyme which Catalyses Racemization of L Acids, *Biochem. Jour.,* **31**: 909–914 (1937).

——— and ———: Part VI. On Some Properties of Racemiase, *Bull. Agr. C Soc. Japan,* **14**: 645–649 (1938).

——— and ———: CCXIII. The Lactic Dehydrogenase of Lactic Acid Bact *Biochem. Jour.,* **32**: 1654–1657 (1938).

——— and ———: Studies on the Enzymes of Lactic Acid Bacteria. I. On F rase of Lactic Acid Bacteria, *Bull. Agr. Chem. Soc. Japan,* **14**: 752–756 (19

Kayser, M. E.: Études sur la fermentation lactique, *Ann. inst. Pasteur,* **8**: 737 (1

Kempe, L. L., H. O. Halvorson, and E. L. Piret: Effect of Continuously Cont pH on Lactic Acid Fermentation, *Ind. Eng. Chem.,* **42**: 1852–1857 (1950).

Kitahara, K., and A. Obayashi: DL-forming Lactic Acid Bacteria, *Jour. Gen. A Microbiol.,* **1**(4): 237–245 (1955).

———, A. Obayashi, and S. Fukui: *Enzymologia,* **15**: 259 (1952).

Koser, S. A., and F. Saunders: Accessory Growth Factors for Bacteria and Rel Microorganisms, *Bact. Revs.,* **2**: 99–160 (1938).

Kozai, Y.: Beiträge zur Kenntnis der spontanen Milchgewinnung, *Zeit. Hyg. In tionskrankh.,* **31**: 337 (1899).

Krueger, K. K., and W. H. Peterson: The Nutritional Requirements of *Lactobac pentosus* 124–2, *Jour. Bact.,* **55**(5): 683–692 (1948).

Leonard, R. H., W. H. Peterson, and M. J. Johnson: Lactic Acid from Fermenta of Sulfite Waste Liquor, *Ind. Eng. Chem.,* **40**: 57–67 (1948).

Needle, H. C., and R. S. Aries: Lactic Acid and Lactates, *Sugar,* December, 1 pp. 32–36.

Nelson, M. E., and C. H. Werkman: Dissimilation of Glucose by Heterofermenta Lactic Acid Bacteria, *Jour. Bact.,* **30**: 547–557 (1935).

——— and ———: Diversion of the Normal Heterolactic Dissimilation by Addi of Hydrogen Acceptors, *Jour. Bact.,* **31**: 603–610 (1936).

——— and ———: The Dissimilation of Pyruvic Acid by *Lactobacillus lycoper Iowa State Coll. Jour. Sci.,* **10**: 141–144 (1936).

Nencki, M.: Die isomeren Milchsäuren als Erkennungsmittel einzelner Spaltpilzar *Zentr. Bakt. Parasitenk.,* **9**: 304 (1891).

Olive, T. R.: Waste Lactose Is Raw Material for a New Lactic Acid Process, *Ch & Met. Eng.,* **43**: 480–483 (1936).

Orla-Jensen, S.: The Lactic Acid Bacteria, *Kgl. Danske Videnskab. Selskabs, Skri Naturvidenskab. math. Afdel.,* Ser. 8, **5**: 51–196 (1919).

———: "Dairy Bacteriology," 2d ed. (in English), McGraw-Hill Book Comp Inc., Blakiston Division, New York, 1931.

———, N. C. Otte, and A. Snog-Kjaer: Der Vitaminbedarf der Milchsäureb terien, *Zentr. Bakt. Parasitenk.,* Abt. II, **94**: 434–447 (1936).

Pan, S. C., W. H. Peterson, and M. J. Johnson: Acceleration of Lactic Acid Ferm tation by Heat-Labile Substances, *Ind. Eng. Chem.,* **32**: 709–714 (1940).

Peckham, G. T., Jr.: The Commercial Manufacture of Lactic Acid, *Chem. Eng. N* **22**: 440–443, 469 (1944).

Pederson, C. S.: The Fermentation of Glucose, Fructose and Arabinose by Org isms from Spoiled Tomato Products, *N.Y. State Agr. Exp. Sta., Tech. Bull.* 1929.

———: The Gas-producing Species of the Genus *Lactobacillus, Jour. Bact.,* **35**: 108 (1938).

———, W. H. Peterson, and E. B. Fred: The Forms of Lactic Acid Produced Pure and Mixed Cultures of Bacteria, *Jour. Biol. Chem.,* **68**: 151 (1926).

Peterson, W. H., and E. B. Fred, *Jour. Biol. Chem.,* **44**: 29–45 (1920).

ord. W. P., E. H. Harris, Jr., C. H. Fisher, and C. O. Willits: Extraction of tic Acid from Water Solution by Amine-solvent Mixtures, *Ind. Eng. Chem.*, 778-781 (1951).

L. T., and H. V. Claborn: Utilization of Lactic Acid, *Ind. Eng. Chem.* (*News* ), **17**: 370 (1939).

and ———: The Production of Pure Lactic Acid, *Ind. Eng. Chem.* (*News Ed.*), 641 (1939).

. E., F. M. Strong, and W. H. Peterson: CCXXIII. Growth Factors for teria. VI. Fractionation and Properties of an Accessory Factor for Lactic d Bacteria, *Biochem. Jour.*, **31**: 1789–1799 (1937).

———, and ———: Pantothenic and Nicotinic Acids as Growth Factors for ctic Acid Bacteria, *Jour. Am. Chem. Soc.*, **60**: 2825 (1938).

———, and ———: Growth Factors for Bacteria. VIII. Pantothenic and otinic Acids as Essential Growth Factors for Lactic and Propionic Acid teria, *Jour. Bact.*, **38**: 293–308 (1939).

E. L. Tatum, and W. H. Peterson: Growth Factors for Bacteria. III. Some tritive Requirements of *Lactobacillus delbrückii*, *Jour. Bact.*, **33**: 207–225 37).

H. R., and L. M. Pruess: Nutrient Requirements of *L. delbrückii* in the Lactic mentation of Molasses, *Jour. Bact.*, **36**: 149–153 (1938).

. E. L., and W. H. Peterson: Fermentation Method for Production of Dextro- ctic Acid, *Ind. Eng. Chem.*, **27**: 1493 (1935).

———, and E. B. Fred: Effect of Associated Growth on Forms of Lactic id Produced by Certain Bacteria, *Biochem. Jour.*, **26**: 846–852 (1932).

———, and ———: Enzymic Racemization of Optically Active Lactic Acid, ochem. Jour., **30**: 1892–1897 (1936).

r, E. O., and L. A. Rogers: Continuous Fermentation in the Production of ctic Acid, *Ind. Eng. Chem.*, **23**: 532–534 (1931).

and B. H. Webb: "By-products from Milk," New York, 1951.

H. G., A. A. Andersen, and C. H. Werkman: Growth-factors for Propionic l Lactic Acid Bacteria, *Proc. Soc. Exptl. Biol. Med.*, **36**: 217–219 (1937).

vard, C. F.: *Chemurgic Dig.*, **9**(6): 9 (1950).

### Some Patents

. E., J. F. Walsh, and H. C. Needle: Fermentation Process of Making White lcium Lactate, U.S. Patent 2,143,359, Jan. 10, 1939.

ll, S. E.: Manufacture of Lactic Acid, U.S. Patent 1,569,221, Jan. 2, 1926.

. L.: Purification of Lactic Acid, U.S. Patent 2,013,096, Sept. 3, 1935.

ım, A. D.: Purification of Lactic Acid, U.S. Patent 2,013,104, Sept. 3, 1935.

. H. C.: Fermentation Process for Producing Lactic Acid and Lactates, U.S. tent 2,143,360, Jan. 10, 1939.

n. W. H., and E. B. Fred: Production of Lactic and Acetic Acids, U.S. tent 1,856,425, May 3, 1932.

rd Brands, Inc.: U.S. Patent 2,032,433, 1936.

, O. M.: Process for Producing Lactic Acid, U.S. Patent 2,009,753, July 30, 35.

. J. F., and H. C. Needle: Lactic Acid Fermentation Process, U.S. Patent 43,358, Jan. 10, 1939.

## CHAPTER 18

# SAUERKRAUT

Sauerkraut is the clean, sound

> . . . product of characteristic flavors, obtained by full fermentation, chiefly la of properly prepared and shredded cabbage in the presence of not less than 2 cent, nor more than 3 per cent salt. It contains, upon completion of the ferm tation, not less than 1.5 per cent of acid, expressed as lactic acid. Sauerkr which has been rebrined in the process of canning or repacking contains not l than 1 per cent of acid, expressed as lactic acid.[1]

The fermentation is a natural one induced by bacteria resident on t leaves of the cabbage. By suitable control procedures involving selecti and cleanliness of materials and regulation of the conditions of ferment tion, an excellent food product is made.

**Varieties of Cabbage Used for Sauerkraut Manufacture.** Sauerkra may be manufactured from many different varieties of cabbage, but t varieties that grow slowly and form closely filled heads are preferred. Seasons, All Head Early, Glory of Enkhuizen, and Flat Dutch are so of the desirable varieties (LeFevre, 1928).

The variety grown will depend in part on local conditions of the clima and soil available.

Fully matured cabbage should be used for sauerkraut manufactu since the use of green cabbage often leads to the production of kraut th is defective in color and in texture.

**Composition of Cabbage.** Various analyses have been given for ca bage (Winton and Winton, 1935). Atwater and Bryant (1906) state th cabbage contains 86 to 94.3 per cent water, 2.9 to 6.4 per cent sug 0.2 to 2.4 per cent protein, 0.1 to 0.7 per cent fat, 0.5 to 1.6 per cent fib and 0.4 to 2.4 per cent ash. Peterson, Fred, and Viljoen (1925), in anal ses of fresh cabbage, found 91 to 93.9 per cent water, 3 to 4.2 per ce sugar, and 0.15 to 0.24 per cent total nitrogen.

**Commercial Manufacturing Procedure.** Preparatory to shreddi sound cabbage is stored in well-ventilated buildings for at least 1 day induce wilting and to obtain a product of more uniform temperatu The wilted cabbage should be cut with the production of the least amou of injury to the shreds, for damage might result in a subsequent softeni of the sauerkraut. The spiral drill of a coring device cuts the core b does not remove it from the cabbage, which is passed on a conveyor to ing table where the outer leaves and any undesirable portions are ed. The cabbage is sliced by machine, and the shreds are trans- to the fermentation vats, some of which have a capacity of at least s of sauerkraut. Salt may be applied evenly to the shredded cab- s it is being distributed in the vat, or it may be spread through oduct in the carts that carry the cabbage from the cutting rooms vats. A round cover is placed over the salted cabbage in each vat a manner that very little of the cabbage is left exposed to the air. ts, usually cement blocks with handles, are placed on the cover to he cabbage under the juices extracted from the shredded and salted t. When the acid content has reached at least 1.5 per cent, a s that may require from 3 to 4 or more weeks, depending on the rature, the fermentation is usually complete.

---

[1] *U.S. Dept. Agr., Service and Regulatory Announcements, Food Drug Adm* No. 2, Revision 5, Nov. 5, 1936.

**ducts of Fermentation.** Juices containing the sugars and other substances are withdrawn from the shredded cabbage by the of the salt. The bacteria that bring about the characteristic sauer- fermentation act upon the sugars and break them down to lactic cetic acids, mannitol, ethyl alcohol, carbon dioxide, and small ts of other products. Esters are formed from alcohol and the c acids.

**roorganisms.** Many types of microorganisms may be found on bbage. Pederson (1931), of the New York State Agricultural iment Station, classifies the more important microorganisms that ffect fermentation into three groups: one group contains those ia which bring about the normal sauerkraut fermentation, one contains spoilage bacteria, and one group contains yeasts.

**irable Types.** The common lactic-acid-producing bacteria that e a normal sauerkraut fermentation may be further divided into ypes: gas-producing cocci, non-gas-producing rods, and gas-produc- ls. These three types are the only ones to develop in the normal lled fermentation (Pederson, 1930). During the first part of the raut fermentation, the gas-producing cocci, *Leuconostoc mesen-* s, predominate, for they find the salt concentration of 2.5 per cent e temperature of 70°F., or below, favorable. These cocci attack gars incompletely and produce lactic and acetic acids, ethanol, tol, and carbon dioxide. They produce the least amount of change protein and in the cellular structure of the sauerkraut. When the ontent increases to 0.7 to 1 per cent, they are destroyed. During development, esters, which impart flavor to the sauerkraut, are d from the acids and alcohol produced by them.

two other types of lactic-acid-producing bacteria do not develop y at first under the conditions of the fermentation, but they gradu- ain ascendency and carry the fermentation to completion. One of types, the non-gas-producing rods, includes *Lactobacillus cucu-* (a probable synonym of *L. plantarum*) and *L. plantarum*, which e lactic acid in large quantities (refer to the section on homo- tative lactobacilli) from a portion of the unchanged sugar and the tol, the latter substance having been produced from sugar by the gas-producing cocci. These lactobacilli are slowly destroyed, owin their lack of resistance to the acid produced.

Fermentation is completed by the third type, *L. pentoaceticus* (a pr able synonym of *L. brevis*), which produces lactic and acetic ac ethanol, mannitol, and carbon dioxide from the remaining sugars. T organism is quite resistant to acid, withstanding an acid content as h as 2.4 per cent.

Table 99, from Pederson's researches, shows analyses of partially completely fermented sauerkrauts.

TABLE 99. ANALYSES OF PARTIALLY AND COMPLETELY FERMENTED KRAUTS*

| Kraut no. | Total acid, per cent | Lactic acid, per cent | Acetic acid, per cent | Acetic acid/ lactic acid ratio | Alcohol, per cent | Mannitol, per cent | Su |
|---|---|---|---|---|---|---|---|
| | | | Partially fermented kraut | | | | |
| 108 | 0.85 | 0.535 | 0.210 | 0.39 | 0.44 | 0.58 | T |
| 109 | 0.98 | 0.615 | 0.237 | 0.38 | 0.32 | 0.54 | T |
| 111 | 0.94 | 0.515 | 0.283 | 0.55 | 0.36 | 0.42 | T |
| 113 | 0.88 | 0.530 | 0.234 | 0.44 | 0.32 | 0.39 | Tr |
| | | | Completely fermented kraut | | | | |
| 109 | 1.67 | 1.268 | 0.268 | 0.21 | 0.62 | Trace | N |
| 111 | 1.78 | 1.304 | 0.318 | 0.24 | 0.47 | Trace | N |
| 113 | 1.67 | 1.183 | 0.324 | 0.27 | 0.65 | Trace | N |

* C. S. Pederson, *N.Y. State Agr. Expt. Sta., Bull.* 595, 1931.

A marked increase in the quantity of lactic acid with but sli increases in the acetic acid and alcohol content indicate that the n gas-producing lactobacilli have dominated during the last stages of fermentation.

The spoilage types of bacteria are usually aerobic and attack proteins, exert but little action toward the sugars, and fail to grow in a media. In the normal fermentation this group fails to gain ascender

Yeasts grow in the presence of oxygen, attack the sugars and lac acid, but rarely develop during the early part of the fermentation. Th may cause pink sauerkraut unless controlled.

**Starters.** The use of starters is not advocated, although under cert circumstances good to excellent results have been secured throu their use.

A starter may consist of a pure culture, such as *L. mesenteroides* *Streptococcus lactis;* of a mixed culture, containing those organisms n mally found in the sauerkraut fermentations; or of sauerkraut juice.

Starters containing pure cultures of the cocci that predominate dur

tial period of the fermentation, or kraut juice, with an acidity of
r cent or less, withdrawn during the early stage of a fermentation,
ring about a normal fermentation. The value of the use of such
s is questionable, according to Pederson (1933).

n the sauerkraut juice contains more than 0.30 per cent of acid,
velopment of *L. mesenteroides* is inhibited and the resultant kraut
inferior quality. The non-gas-producing rods predominate under
onditions.

sauerkraut juices, juices containing more than 0.30 per cent acid,
ltures in which the lactobacilli (*L. cucumeris* and *L. pentoaceticus*)
inate usually produce sauerkrauts of a poorer quality. For these
s, sauerkraut manufacturers are advised to wash thoroughly and
any vats in which a sauerkraut fermentation is contemplated and
h a fermentation has just been completed.

e factors are of much importance in regulating the fermentation
age: the concentration of the salt, the covering of the sauerkraut,
e temperature of fermentation.

. Salt serves several important functions in the sauerkraut fer-
ion. It draws the juices out of the cabbage; it favors a lactic acid
tation, the acid of which checks putrefaction; it contributes to
vor; and it has much to do with firmness of the final product.

greater the amount of salt used, the more effective will be the
ion of juices, and the firmer will be the shredded cabbage leaves.
igh concentrations of salt will inhibit the desirable fermenting
ia to some extent. It becomes necessary, therefore, to select a con-
tion that will permit a normal fermentation and give a firm, high-
sauerkraut. A salt concentration of 2.5 per cent is favored by many
rcial sauerkraut manufacturers—2.5 lb. of salt per 100 lb. of
e; but some favor a concentration of 2.25 per cent.

lage types are not inhibited to any great extent until a salt con-
5 to 7 per cent is used. A high concentration of salt, therefore,
be depended on to prevent spoilage. This concentration of salt
eover, prohibitive for the sauerkraut fermentation.

salts used in the fermentation should be of a known, pure grade
om calcium and magnesium salts and should be measured by weight
than by volume, since densities vary for different salts. Salt
be thoroughly mixed with the shredded cabbage.

916, Henneberg advanced the opinion that salt is added to cabbage
o withdraw juice. Hof (1935) concluded, as the result of experi-
in which 2.5 per cent salt was added to cabbage in one case and
but no salt in the second case, that the most important reason for
salt to cabbage was to promote the formation of juice. The sauer-
made without salt was much softer than that prepared with salt.
(1930) states that sauerkraut may be made by adding water,
t salt, to the cabbage. Such sauerkraut is very soft, however.

salt concentration of a sauerkraut may be determined by titrating
le of the juice with 0.1 *N* silver nitrate, using potassium chromate
indicator.

**Covering the Sauerkraut.** The covering of sauerkraut during fe tation is necessary to exclude oxygen, which favors the developm yeasts and spoilage types of bacteria. (See the section on sauer spoilage.) Weights are placed on the cover in a vat to force the extr juices over the uppermost layer of sauerkraut. Muslin cloth is some used to aid in maintaining moisture in the product at the top o vat.

**Plastic Covers for Tanks.** It has been known for some time tha use of plastic films to cover the top of cucumber salt-stock ferme aided in the prevention of undesirable mold and yeast growth on th face. Such coverings have been used very successfully in the Depart of Food Technology of the Massachusetts Institute of Techno Recently a method of curing sauerkraut in open-top tanks, by cov the top with a plastic sheet impervious to water, gas, and air, has patented by Kauble (1957). A slack center is formed by the sheet, the gases evolved during fermentation are allowed to escape. Flo weighting agents may be applied to the film.

**Temperature.** The optimum temperature for the sauerkraut fer tation is believed to be 65 to 70°F. (18.3 to 21.1°C.), while the ran 60 to 70°F. (15.6 to 21.1°C.) is satisfactory, according to Ped (1930). Any temperatures below 60°F. (15.6°C.) retard the fermenta As was stated earlier, *L. mesenteroides* grows well at temperatures b 70°F. (21.1°C.), but, at 60°F. (15.6°C.) or below, the fermentation usually cease after the production of about 1 per cent of acid, since lactobacilli fail to develop.

Experience has shown that the use of high temperatures for the mentation is also undesirable. The use of temperatures around 8 (30°C.) favors the development of the lactobacilli but retards the de opment of *Leuconostoc,* which is primarily a flavor-producing organ In order to maintain cabbage at this temperature, artificial heating be employed. If the building is heated to above 86°F. (30°C.), as w be necessary, spoilage is likely to be incurred.

**Spoilage of Sauerkraut.** Aside from purely aesthetic reasons importance of maintaining a sauerkraut factory in a high state of cle ness is emphasized by a study of the following causes of spoilage.

*Dark sauerkraut* is not uncommon and may be caused by a nu of factors. Exposure of the sauerkraut to air may produce a dark pro Too much salt in certain cases, the result of uneven distribution, ca darkening of sauerkraut ("burned" sauerkraut), since normal fer tors but not spoilage types of bacteria are inhibited by high salt con trations. Darkened sauerkraut may be induced by the use of excess high temperatures during fermentation, conditions that favor the deve ment of abnormal types of bacteria. The presence of iron and ta (from wood), or the surface rotting of sauerkraut may also produ darkened product. When the fermenting product is improperly weig and the juice fails to cover or moisten the surface, aerobic bacteria yeasts are likely to produce a darkening of the sauerkraut at the sur owing to the faulty fermentation that they cause.

: *sauerkraut* is caused by certain kinds of yeast. The types found erkraut develop only when air is present. Hence they are usually in the juice at the surface of the sauerkraut, occasionally as a scum, when not well covered, or in the air pockets formed between les of the vat and the shredded cabbage, especially when the salt bbage have been unevenly mixed. Sugar and, in some cases, acid e attacked by them. They also produce a pigment that may vary pink to an intense red in color. In completely and properly fer- l normal sauerkraut these organisms do not usually develop.

growth of pink yeasts is favored by factors that inhibit the normal nce of bacterial growth (Pederson and Kelly, 1938). Thus a high rature of fermentation, a salt concentration greater than normal, reased acid content, and a cabbage of low nitrogen content may the growth of the yeasts producing a pink or red color in sauer- (Peterson et al., 1927).

*sauerkraut* may be due to the use of too little salt, high tempera- faulty fermentation, or exposure to the air. The use of insufficient ay produce a soft sauerkraut because it fails to draw enough juice he cabbage or because it permits the lactic-acid-producing rods to p. A high temperature favors the growth of lactobacilli at the ning of the fermentation rather than in their proper sequence, with sult that the structure of the cabbage leaf is broken down to some . In such cases, the cocci fail to develop properly. Improper clean- a vat that has been packed previously, combined with high tem- ires, favors the predominance of those types of bacteria which are only found at the end of the fermentation. These bacteria have, viously stated, a greater ability to weaken the structure of the ge. Softening of sauerkraut by bacteria or yeasts growing on the e of a product that has undergone a normal fermentation is not on.

*y sauerkraut* is caused usually by the rapid growth of certain of *L. cucumeris* and *L. plantarum*, especially at raised tempera- Such sauerkraut is edible but of an objectionable character. The nay sometimes be dissolved by canning or cooking.

*ted sauerkraut* may be caused by molds, yeasts, bacteria, or, once hile, fruit flies. A heavy muslin cloth placed over the surface of redded cabbage and pushed down the sides of the vat at the time king it may be of considerable value in preventing rot. The cover ced directly over the cloth with enough weights on top of it to press the cabbage without causing the juice to flow over the cloth. Since urface of the fermenting sauerkraut is kept moist the growth of irable types of bacteria will be inhibited. Flies will be excluded by oth.

*-flavors* may result from changes in the normal sequence of bacterial pment, or from too rapid a fermentation.

iethod for preventing the development of aerobic organisms during uerkraut fermentation has been patented by Harrison (1944). The ntation vessel is covered with a gastight closure, equipped with a release valve. The carbon dioxide produced during fermentation d out the air. After the fermentation is complete, an atmosphere of ca dioxide may be maintained by adding the gas from a pressure tank.

**Cabbage for Sauerkraut.** Table 100 gives information concernin production of cabbage for sauerkraut.

TABLE 100. CABBAGE FOR KRAUT: 1946 WITH COMPARISONS *

| State | Acres | | | | Production, tons | | | |
|---|---|---|---|---|---|---|---|---|
| | 10-year average | | 1945 | 1946 | 10-year average | | 1945 | |
| | 1927–1936 | 1935–1944 | | | 1927–1936 | 1935–1944 | | |
| New York ...... | 6,280 | 6,530 | 9,200 | 8,300 | 58,700 | 63,800 | 99,300 | 12 |
| Ohio ............ | 2,220 | 1,840 | 2,200 | 2,600 | 18,900 | 14,500 | 19,800 | 2 |
| Indiana ......... | 1,410 | 1,310 | 800 | 600 | 8,600 | 7,800 | 5,600 | |
| Illinois ......... | 670 | 460 | 500 | 680 | 4,200 | 2,900 | 4,000 | |
| Michigan ........ | 1,310 | 900 | 400 | 300 | 9,800 | 6,300 | 4,400 | |
| Wisconsin ...... | 4,640 | 4,760 | 5,800 | 6,300 | 34,500 | 34,700 | 65,000 | 6 |
| Minnesota ....... | 330 | 200 | 300 | 200 | 2,700 | 1,400 | 2,400 | |
| Colorado ....... | 330 | 250 | 280 | 240 | 3,600 | 2,600 | 3,100 | |
| Washington ..... | 310 | 330 | 400 | 370 | 2,700 | 3,400 | 3,700 | |
| Other states † ... | 1,660 | 2,180 | 2,850 | 2,660 | 11,000 | 15,000 | 26,000 | 2 |
| Total all states | 19,160 | 18,760 | 22,730 | 22,250 | 154,700 | 152,400 | 233,300 | 26 |

* U.S. Department of Agriculture, "Agricultural Statistics, 1947," 1948.

† "Other states" includes Florida, Iowa, Maryland, Missouri, New Jersey, N Carolina, Oregon, Pennsylvania, Tennessee, Texas, Utah, and Virginia.

An examination of this table indicates that New York produces n than one-third of all the cabbage used for sauerkraut manufacture, w Wisconsin produces more than one-fifth. Approximately three-fifth the total quantity of cabbage is thus produced by New York and Wis sin. Ohio is the third largest producer of cabbage for sauerk manufacture.

Other information of interest concerning the statistics of cabb production for sauerkraut manufacture and the location of plants be found in *Technical Bulletin* 646, published by the U.S. Departmen Agriculture (Spangler, 1938).

### References on Sauerkraut

Anonymous: Making and Canning Sauerkraut, *Food Inds.*, **17**(10): 94–95 (1945).
Atwater, W. O., and A. P. Bryant: The Chemical Composition of American F Materials, *U.S. Dept. Agr., Bull.* 28, 1906.
Bachmann, F. M.: Growth of *Clostridium botulinum* in Fermented Vegetables, J *Infectious Diseases*, **34**: 129 (1924).
Bitting, A. W.: "Appertizing," Trade Pressroom, San Francisco, 1937.
Brunkow, O. R., W. H. Peterson, and E. B. Fred: The Influence of Certain Fa

n the Chemical Composition of Sauerkraut, *Jour. Am. Chem. Soc.*, **43**: 2241 21).

———, and ———: A Study of the Influence of Inoculation upon the Fermen- on of Sauerkraut, *Jour. Agr. Research*, **30**: 955 (1925).

. E.: Bakteriologische und chemische Studien über Sauerkrautgärung, *Arch. g.*, **29**: 56 (1897).

F. W.: The Science of Making Sauerkraut, *Mich. Agr. Expt. Sta., Quart l.*, **9**: 50 (1926).

. B., and W. H. Peterson: The Production of Pink Sauerkraut by Yeasts, *r. Bact.*, **7**: 257 (1932).

M.: "Meine Diät," Ullstein, Berlin, 1930, p. 96.

n, W. H.: U.S. Patent 2,345,814, Apr. 4, 1944.

erg, W.: Zur Kenntnis der milchsäurebakterien, *Zeit. Spiritusind.*, **26**: 226, , 255, 257, 270, 277, 288, 291, 302, 315, 329, 341, 343 (1903).

Das Sauerkraut, *Deut. Essigind.*, **20**: 133 (1916).

: Investigations Concerning Bacterial Life in Strong Brines, *Rec. trav. botan rl.*, **34**: 93–173 (1935).

, C. H.: Improving the Quality of Kraut, *Canner,* **72**(part 2): **71** (1931).

, E.: Use of Pure Cultures as "Starters" in the Preparation of Sauerkraut, *ner,* **48**(8, part 2): 176 (1919).

Sauerkraut Production Controlled by Heat with the Report of an Experi- nt, *Canner,* **50**(10, part 2): 161 (1920).

Bacteriology of Sauerkraut and Pickles, *Chem. Age* (*N.Y.*), **30**: 24 (1922).

Standardized Methods in Kraut Production, *Canning Age*, 1925, pp. 229, , 237.

The Commercial Production of Sauerkraut, *U.S. Dept. Agr., Circ.* 35, June, 8.

kie, J. E.: Types of Spoilage in Canned Sauerkraut, *Canning Age*, 1927, 40.

ll, C. E.: "Microbiology," McGraw-Hill Book Company, Inc., Blakiston Divi- n, New York, 1921.

, E. A., W. H. Peterson, E. B. Fred, and W. E. Vaughn: Relation of Tempera- e of Fermentation to Quality of Sauerkraut, *Jour. Agr. Research*, **39**: 285 29).

, H. B., E. B. Fred, W. H. Peterson, J. E. McConkie, and W. E. Vaughn: ation of Temperature to Rate and Type of Fermentation and the Quality of nmercial Sauerkraut, *Jour. Agr. Research,* **35**: 1021 (1927).

n, C. S.: Floral Changes in the Fermentation of Sauerkraut, *N.Y. State Agr. t. Sta., Tech. Bull.* 168, 1930.

Sauerkraut, *N.Y. State Agr. Expt. Sta., Bull.* 595, 1931.

The Relation between Temperature and the Rate of Fermentation of Com- cial Sauerkraut, *N.Y. State Agr. Expt. Sta., Bull.* 614, 1932.

The Effect of Inoculation on the Quality, Chemical Composition, and Bac- l Flora of Sauerkraut, *N.Y. State Agr. Expt. Sta., Tech. Bull.* 216, 1933.

Controlling the Quality of Kraut during Manufacture, *Food Inds.*, **19**: 212, -780 (1947).

and C. D. Kelly: The Quality of Commercial Sauerkraut, *N.Y. State Agr. pt. Sta., Bull.* 613, 1932.

and ———: Development of Pink Color in Sauerkraut, *Food Research,* **3**: (1938).

G. L. Mack, and W. L. Athawes: Vitamin C Content of Sauerkraut, *Food earch,* **4**: 31 (1939).

n, W. H., and E. B. Fred: An Abnormal Fermentation of Sauerkraut, *Zentr. t. Parasitenk.*, Abt. II, **58**: 199 (1923).

———, and J. A. Viljoen: Variations in the Chemical Composition of Cab- e and Sauerkraut, *Canner,* **61**(4): 19 (1925).

H. B. Parmele, and E. B. Fred: Some of the Factors Which Influence the nposition of Cabbage and Their Relation to the Quality of Sauerkraut, *Soil* , **24**: 299 (1927).

Prescott, S. C., and B. E. Proctor: "Food Technology," McGraw-Hill Book pany, Inc., New York, 1937.

Pruess, L. M., W. H. Peterson, and E. B. Fred: Gas Production in the Mak Sauerkraut, *Ind. Eng. Chem.*, **20**: 1187 (1928).

Round, L. A.: Kraut Investigations, *Canner*, **44**(9, part 2): 113 (1917).

Spangler, R. L.: Marketing Commercial Cabbage, *U.S. Dept. Agr., Tech. Bul* October, 1938.

Wehmer, C.: Die Sauerkrautgärung, *Zentr. Bakt. Parasitenk.*, Abt. II, 10 (1903).

———: Untersuchungen über Sauerkrautgärung, *Zentr. Bakt. Parasitenk.*, Ab **14**: 682 (1905).

Winton, A. L., and K. B. Winton: "The Structure and Composition of Foods," Wiley & Sons, Inc., New York, 1935, Vol. II.

## CHAPTER 19

# PICKLES

les are

mature cucumbers properly prepared, without taking up any metallic
nds other than salt, and preserved in any kind of vinegar, with or without
Pickled onions, pickled beets, pickled beans, and other pickled vegetables
etables, prepared as described above, and conform in name to the vegeta-
d.[1]

**sification of Pickles.** Owing to modifications in methods of prep-
, there is a large variety of pickles. However, these may be con-
tly grouped as dill, sour, and sweet pickles, according to the
cation prepared by Fabian and Switzer (1941), which follows:

l pickles
- Fermented dill pickles
  - a. Genuine dill pickles
  - b. Genuine Kosher dill pickles
  - c. Overnight dill or fresh fermented dill pickles
  - d. Overnight or fresh fermented Kosher dill pickles
  - e. Polish dill pickles
- Unfermented dill pickles made directly from fresh cucumbers
  - Fresh or pasteurized dill pickles
  - Iceberg or quartered dill pickles
- Dill pickles made from salt stock
  - Processed imitation or summer dill pickles
  - Processed imitation or summer Kosher dill pickles
  - Pasteurized processed dill pickles
  - Pasteurized processed Kosher dill pickles

pickles
- Sour pickles
  - Plain sour pickles
  - Sliced or hot sour pickles
- Mixed sour pickles
  - Mixed unspiced
  - Spiced or hot mixed pickles
  - Mixed chutney
- Relish, chowchow, etc.
  - Chowchow

et pickles
- Plain sweet pickles
  - Standard sweet pickles
  - Midget sweet pickles
  - Burgerkins

---

*Dept. Agr. Service and Regulatory Announcement, Food Drug Admin.,* Revision 5, Nov. 5, 1936.

    d. Slices, chips or wafers
    e. Candied chips
    f. Sweet dill pickles
    g. Bread and butter or old-fashioned pickles
    h. Peeled pickles
  B. Mixed sweet pickles
    a. Plain mixed
    b. Mustard pickles
    c. Jamaica pickles
  C. Relish or chopped sweet pickles
    a. Plain relish
    b. Spread relish
    c. India relish
    d. Piccalilli
    e. Fruit relish
    f. Mexican relish
    g. Vegetable relish

Although almost any cucumbers free from disease may be used to r pickles, it is customary to select certain varieties, such as Snow's Pe tion, Chicago Pickling, or Boston Pickling, which are preferabl account of their size, shape, or keeping qualities.

**Preliminary Operations.** The harvesting, inspection, grading, usually the washing of cucumbers are preliminary steps in the manu ture of most kinds of pickles. The cucumbers are harvested at the pr stage of maturity, usually when rather small, green, and firm. They inspected for defects, and those unsuitable on account of moldines other form of deterioration are discarded, since mold growth, eve slight amounts, may produce marked off-flavors in the pickled proc especially if it is of the "bread-and-butter type," and decomposed cuc bers may infect pickling tanks.

Cucumbers may be graded for size on the basis of the number requ to fill a 45-gal. cask. For example, if the cask holds 800 cucumbers, are classed as large; if 1,200, medium; and if 3,000, small (Jones Etchells, 1943).

Although cucumbers are not always washed prior to processing, essential to clean thoroughly those used in the manufacture of un mented pickles. Soaking of the cucumbers in clean water aids in loo ing the soil. Thorough washing removes any adhering dirt and gener reduces the number of microorganisms.

Cucumbers used for the production of salt-stock or dill pickles placed in special vats and covered with brine. The vats, which are ally constructed of cypress, fir, pine, or redwood, are generally 6 to in depth and up to 16 ft. in diameter, and hold from 85 to 1,600 or r bu. of cucumbers. They are usually located in the open but may erected in buildings. They must be thoroughly cleaned before use; they may be soaked, prior to washing, either in water containing a n mum of 50 p.p.m. of chlorine or in some other suitable germicide w will not impart odor, color, or toxicity to the tank or cucumbers. A top is placed over the cucumbers, and the brine is added until the fo is submerged.

**ng.** There are in general two methods for adding brine to cucum-
lthough there are many variations of these methods, and although
may be dry-salted. In one method, a low-salt curing process, the
ers are added to a tank partially filled with brine, which gives a
eter reading (refer to page 363) of 30° (nearly 8 per cent sodium
e). This concentration of salts is considered to be the lowest that
used with safety under ordinary conditions. Salt is then added in
portion of 9 lb. for each 100 lb. of cucumbers in the tank. There-
ufficient salt is added to raise the brine 3° salinometer each week
final reading of 60° (15.9 per cent) is obtained. Fermentation
ring are rapid by this method, but there is greater danger of unde-
bacteria predominating and the pickles are not likely to be so
when a higher initial concentration of salt is used.

he second procedure, a brine containing 10.6 per cent salt (40°
neter) is used. The cucumbers are placed in the brine, and salt is
in the proportion of 9 lb. per 100 lb. of cucumbers. The salt con-
tion of the brine is increased 2° salinometer each week until it
s 50° and thereafter 1° each week until a final reading of 60°
neter is obtained. Fermentation and curing by this procedure are
, but there is less danger of spoilage types of microorganisms pre-
ating, and the pickles are firmer than by the low-salt curing method.
rocedure is especially desirable where temperatures are likely to be
ely high, as in the South. It is also advantageous in cold regions
the higher initial concentration of salt prevents freezing of the
ts of the tank.

s essential that very hard waters should not be used for pickle
. The salt should be of a high quality and should contain less than
ent of sodium, calcium, or magnesium carbonates or bicarbonates,
ese salts will neutralize the acid produced by the lactic-acid-
ing bacteria and favor the development of proteolytic types.

the practice with some picklers to add starters from actively fer-
ng tanks or other containers. Such starters contain acid-producing
ia of a desirable type. These bacteria are accustomed to high con-
tions of salt, and under such circumstances the acidity of the brine
crease rapidly. Since cucumbers contain less sugar than sauerkraut
ften possess more mixed types of microorganisms, the use of a
r is frequently highly desirable. Sugar to the extent of approxi-
y 1 per cent may be added to favor desirable fermentation, espe-
if the pickles are deficient in sugar, according to LeFevre.

ce the tank has been filled, the curing and fermentation should be
ully controlled. The pickles should be kept well covered by brine
l times by the use of false covers or heads, which are fastened in
. The strength of the brine should be checked daily. After a tank
een filled and salt has been added, it is usually desirable to pump the
from the bottom of the tank to the top in order to ensure a uniform
ntration of the brine. Any scum containing yeasts or molds that
on the surface of the brine should be removed periodically to
nt consumption of the acids formed by fermentation.

**Influence of Salt.** Cucumbers contain about 95 per cent of w When cucumbers are in a tank surrounded by salt in the form of they lose water by osmosis. Dissolved in the water are sugar, so proteins, minerals, and other substances, which are used as food b lactic acid bacteria and any other microorganisms present, provide conditions are favorable.

The brine is diluted and weakened as the result of the water withdrawn from the cucumbers. At the same time some of the sa absorbed by the cucumbers. Under these circumstances the salt con tration of the brine may become so reduced that spoilage types of bac may grow and perhaps predominate unless more salt is added to main a fairly high concentration of the brine.

The curing and fermentation of cucumbers, a process that is influe by the salt concentration of the brine, the temperature, the size o cucumbers, and the control of the scums (molds, yeasts), may req 6 to 9 weeks. During the process, the flavor, texture, and color of cucumbers change. The cucumbers are converted from a pale gree an olive or dark green in color. The color change extends through the pickle. Thus, if the pickle is opaque or white internally, it is properly cured. Lactic acid bacteria are responsible for these chan while salt is the most important factor in controlling the fermentat Salt tends to preserve the chlorophyll and other constituents of the pic

**Changes during the Cucumber Fermentation.** Complex physi biochemical, and microbiological changes take place during the fermen

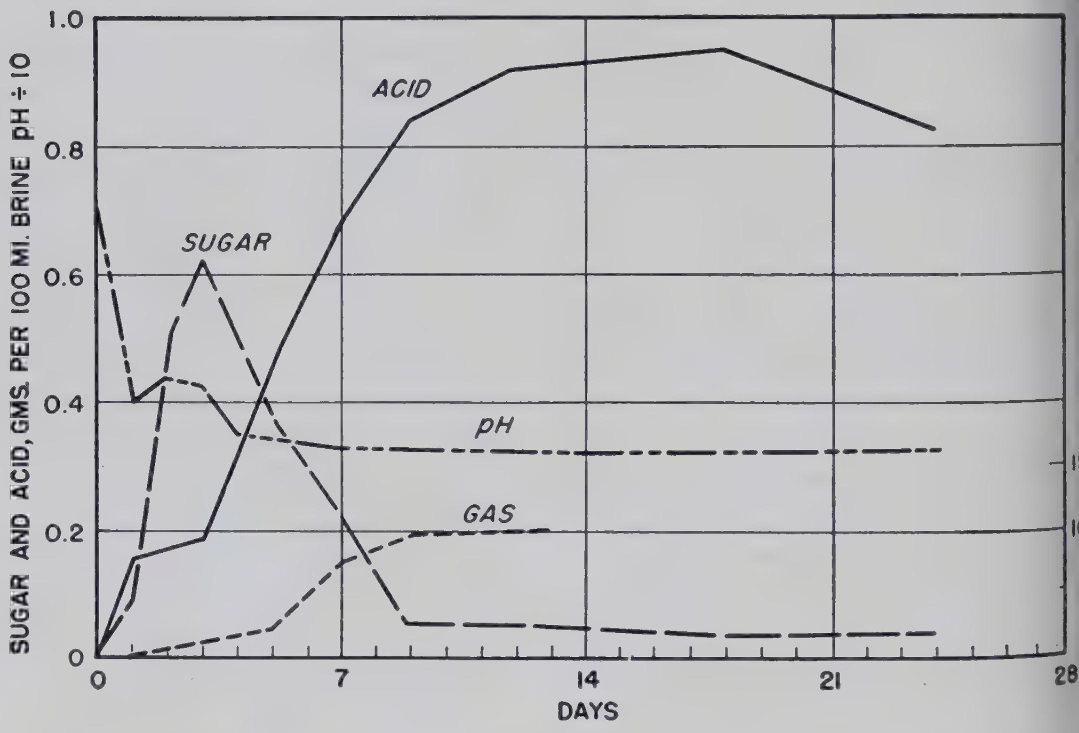


FIG. 56. Relationship of changes in titrable brine acidity, brine sugar concentrat and gas evolution from brine surface for a typical dill fermentation (acidity ca lated as lactic acid, sugar concentration as reducing sugar). [*Courtesy of I. D. J and J. L. Etchells, Food Inds.*, 15(1): 62 (1943).]

cucumbers. Some of the changes taking place have already been ed; others are described in the following sections.

*hemical Changes.* The physical and chemical changes taking n the cucumber fermentations have been described by Jones and s. The fermentations observed were carried out during the sum- nths in vats of 85-bu. capac- d of 6-ft. diameter. These rected outdoors and located tern North Carolina. The re filled with cucumbers and o a depth of 4½ ft. Obser- s were made in respect to sugar, acid, pH, and gases. 56 shows the relationship of actors in a typical dill-pickle ntation. Physical and chemi- hanges, though differing in tude, are similar in the curing h dill pickles and salt stock. workers have studied the in- of three different initial concentrations on fermenta- These were designated, on the of their initial concentrations, 40, and 60° brines. The 20° was maintained at a concen- n of 20° salinometer during st week and thereafter in- d 10° per week to a final con- tion of 60°. The 40° brine ept at 40° during the first and thereafter increased 5° veek until a concentration of as obtained. The 60° brine ade up to 60° and maintained at concentration throughout periments. The concentration ar in the brine was expressed rams of reducing sugar per ml. of brine; that of the acid, n was determined by titration, as grams of lactic acid per 100 ml. ine.

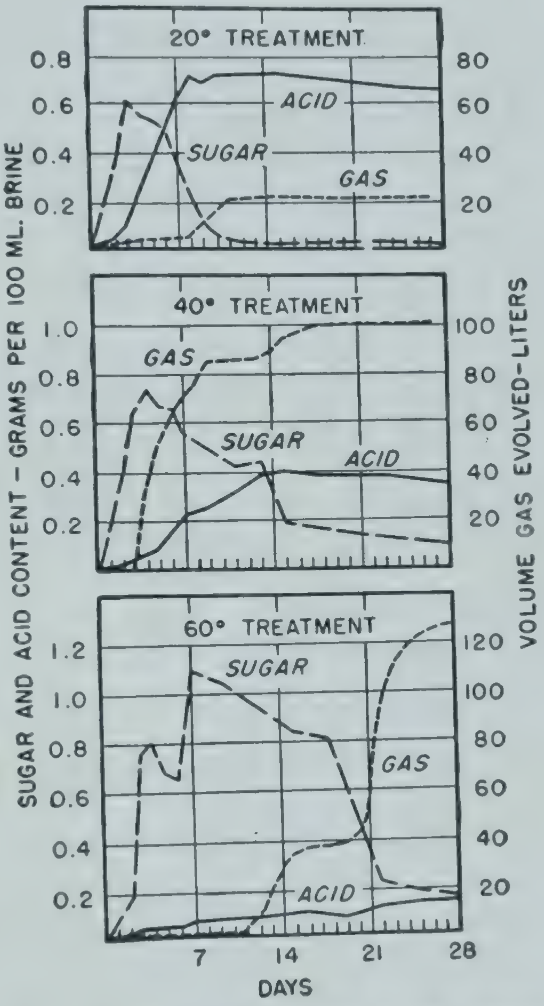


Fig. 57. Changes in brines from experimental lots salted at 20, 40, and 60° salinometer initial salt concentration. These results represent a single experiment during curing. [*Courtesy of I. D. Jones and J. L. Etchells, Food Inds.,* 15(1): 62 (1943).]

rves indicating the changes in the concentrations of sugar, acid, and nes of gas evolved are shown in Fig. 57. Several conclusions may be n as a result of the study of these figures. For example, it may be ved that, as the initial concentration of salt is increased, it requires ger time for the production of the maximum amount of sugar in the . However, the maximum concentration of sugar in the brine increases when the initial concentration of the brine is increased. formation, chiefly by lactic acid bacteria, is most rapid and greatest a lowest brine concentration and decreases progressively as the initial centration of brine is increased. The breaks in the curves for gas pro tion in the 40 and 60° brines are due to two different but concurrent mentations, according to Jones and Etchells. The gas produced du the first part of the fermentation is due to gas-forming bacteria and sists of hydrogen and carbon dioxide, while that produced during second part of the fermentation is evolved by yeasts and consists pri pally of carbon dioxide.

It may be concluded that the use of brines of low initial concentra favors the production of acid by the lactic acid bacteria and only r tively small amounts of gas, whereas the use of brines of high in salinity retards both the rate and quantity of acid formation and fa vigorous gas production.

Studies made by Jones and Etchells over a 4-year period on the c tents of 28 vats indicated that high initial salinities favor the produc of bloaters (hollow cucumbers). Table 101 shows their findings.

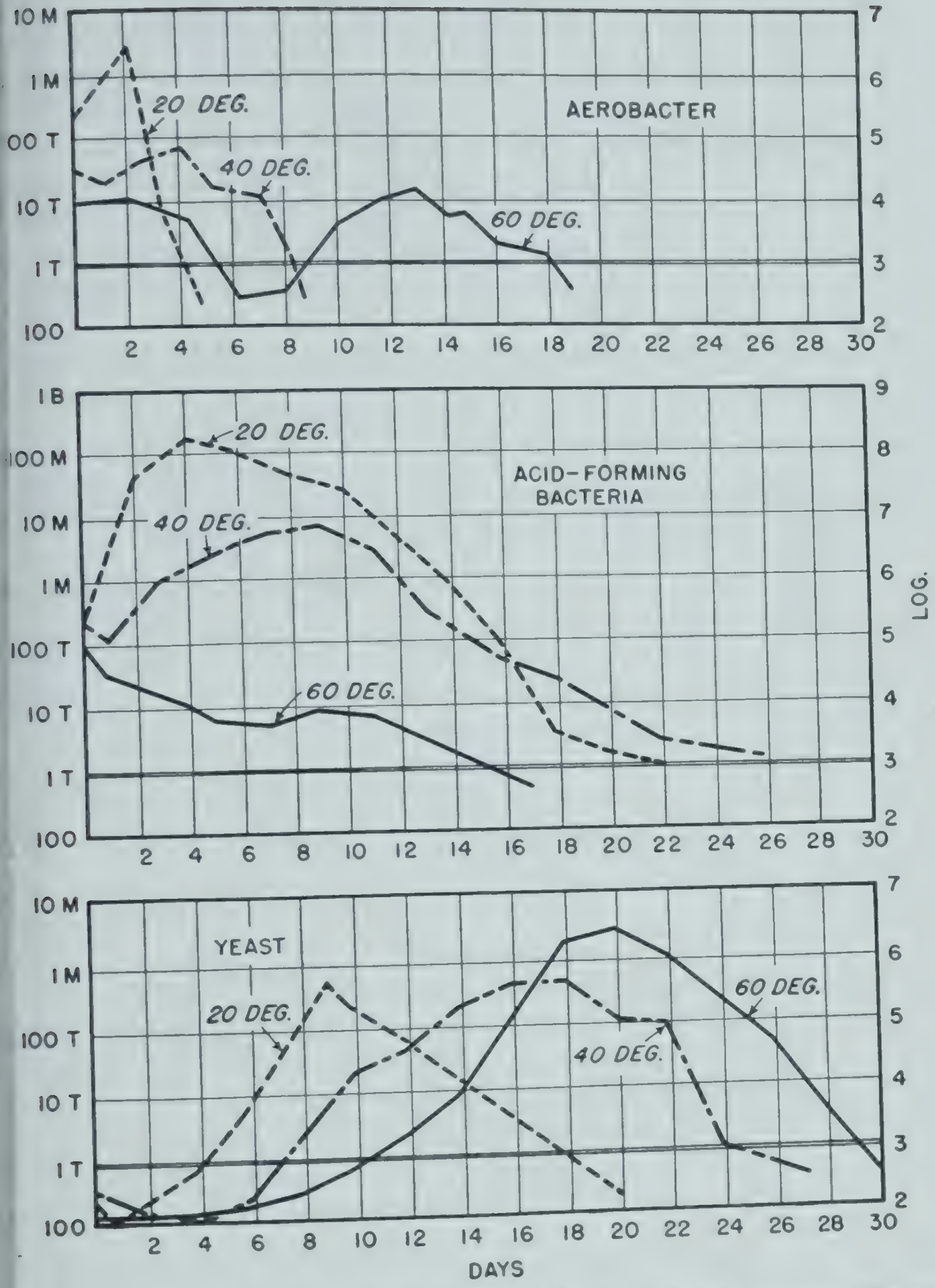
TABLE 101. RELATION BETWEEN SALTING TREATMENT AND PERCENTAGE OF BLOA FORMED DURING CURING OF SALT STOCK *

| Salting Treatment | Bloaters Form Per Cen |
|---|---|
| 20° | 6.5 |
| 40° | 22.9 |
| 60° | 43.6 |

* I. D. Jones and J. L. Etchells, *Food Inds.,* **15**(1): 62 (1943).

*Microbiological Changes.* At least three different types of fermen tion may occur in the brine during the curing process. These are fermentation caused by the acid-formers, which produce lactic acid preservative; the fermentation caused by species of the genus *Aerobac* which is characterized by the production of carbon dioxide and hydro gases; and the fermentation brought about by yeasts, which is dist guished by the production of carbon dioxide gas and ethyl alcohol. Th fermentations are due to microorganisms that occur naturally on cucumbers or in the soil adhering to them. Fermentations comme shortly after the addition of the brine to the cucumbers and continue 2 to 6 weeks or longer.

Reference has already been made, in the foregoing section on b chemical changes, to experiments carried out by Etchells and Jones a pickling plant in eastern North Carolina. These investigators also det mined the influence of three different initial brine concentrations on numbers of acid-forming bacteria, yeasts, and hydrogen-forming bacte of the genus *Aerobacter*. They used the 20, 40, and 60° brines alrea described. The fermentations were carried out principally in 85- vats, in which the brine temperature during the period of the experime was approximately 80°F. Figure 58 presents their data concerning



3. Fermentation trends in 20, 40, and 60° salinometer brines. [*Courtesy of* *tchells and I. D. Jones, Food Inds.*, **15**(2): 54 (1943).]

biological populations of the brines at different initial salinities. ts of microorganisms are plotted logarithmically.

a result of their investigations, Etchells and Jones drew certain usions. The fermentation by the *Aerobacter* group usually occurs in )° brines, gas evolution being vigorous; it may or may not occur in )° brine; and it is brief in duration if it occurs in the 20° brine. The vely weak *Aerobacter* fermentation in the 20° brine is attributed to pid growth of the acid-formers which inhibit the development of the roducing bacteria. The acid-formers grow best in the 20° brine and poorest in the 60° brine. The combination of acid and salt app restrict the activities of this group. Active yeast fermentation place at all the brine concentrations.

The influence of adding sugar to brines has been studied by Ve and his associates (1941). They found that the addition of su brines at the beginning of or during active fermentation resulted increase in the number of acid-formers but that, if sugar was toward the end of active fermentation, it produced no appreciable c The addition of sugar to brine increased also the number of yeasts a amount of gas evolved (Veldhuis et al., 1941).

A summary of information concerning the general relationshi fermentations caused by species of *Aerobacter*, acid-forming bacteri yeasts in the 20, 40, and 60° brines is presented in Table 102.

TABLE 102. GENERAL RELATIONSHIPS IN FERMENTATIONS DUE TO *Aerobacter*, FORMING BACTERIA, AND YEASTS IN 20, 40, AND 60° BRINES *, †

| Organisms | Brine concentration, ° salinometer | General type of fermentation | Fermentation activity | Chief products formed | Approximate start of fermentation ‡ | App m dur of men |
|---|---|---|---|---|---|---|
| *Aerobacter* .... | 20 | Flash-gaseous | Slight, may be absent | Hydrogen and $CO_2$ | 1st day | 2–3 |
| | 40 | Gaseous | May or may not be active | Hydrogen and $CO_2$ | 1st–3d day | 1 w |
| | 60 | Gaseous | Usually active | Hydrogen and $CO_2$ | 7th–8th day | 1 w |
| Acid-forming bacteria ..... | 20 | Acid | Very active | Lactic acid | 1st day | 3 w |
| | 40 | Acid | Active | Lactic acid | 2d–3d day | 3 w |
| | 60 | Acid | Very slight | | | |
| Yeasts ........ | 20 | Gaseous | Active | $CO_2$ and alcohol | 3d–5th day | 2 w |
| | 40 | Gaseous | Active | $CO_2$ and alcohol | 7th day | 17 d |
| | 60 | Gaseous | Active | $CO_2$ and alcohol | 11th–12th day | 17–2 |

* J. L. Etchells and I. D. Jones, *Food Inds.*, **15**(2): 54 (1943).

† Brine temperature about 80°F. during the fermentation period.

‡ After heading tanks.

The *Aerobacter* fermentation has been studied by Etchells, Fabian, Jones (1945).

Pederson and Ward (1949), also, studied the effect of salt on bact logical and chemical changes in fermenting cucumbers. Aerobic bact such as species of *Flavobacterium*, *Achromobacter*, and *Pseudom* which are proteolytic and do not produce acid, were found early i fermentation. Their development should be inhibited.

Although results varied from year to year, lactic acid bacteria always responsible for the major changes in the cucumbers. Lactic bacteria found included *Leuconostoc mesenteroides*, which was pr early in the fermentation only and in low-salt brines; *Streptoc faecalis*, a salt-tolerant organism, which played an important part in fermentations; *Pediococcus cerevisiae*, a high acid-producer and tolerant coccus, which was also important in some fermentations; *L bacillus brevis*, a gas-producing rod, which was somewhat variab ence and growth; and *L. plantarum*, which occurred most fre- y and which was found during the later stages of almost all the tations.

tiplication of the microorganisms and formation of acid were most in 2 to 5 per cent concentrations of salt. Changes in the texture cucumbers, or degree of curing, were quickest in the brines of low salt contents. The rate of fermentation was retarded and the num- f undesirable microorganisms made up a larger proportion of the numbers of microbes when the salt content was initially 7.5 per r higher.

rson and Albury (1950) reported upon the effect of temperature on iological and chemical changes in fermenting cucumbers. On the f their studies, they stated that temperatures of 75 to 86°F. were um for the fermentation and curing of cucumbers. At temperatures to 50°F., *L. mesenteroides* and low-acid strains of *L. plantarum* led. At temperatures of 65°F. and higher, *Strept. faecalis*, *P. siae*, *L. brevis*, and typical strains of *L. plantarum* were more active. °F., the initial rate of fermentation was highest.

**litions to the Cucumber Fermentation.** Studies were carried out gheb and Fabian (1957) on a semicommercial and laboratory basis two seasons to determine the effect of adding sugar, amino acids, orn-steep liquor to the cucumber brine. Acid production was not ated by the addition of sugar (0.5 to 1 per cent) or cystine and phan (0.0025 per cent each). However, the addition of corn-steep enhanced the formation of acid and increased utilization of the in the brine.

**sts.** Yeasts are always associated with the brine fermentation of bers. They are directly concerned with the formation of bloaters, the two chief types of spoilage occurring during the brining opera- They undoubtedly contribute to the flavor of the product.

yeasts found in cucumber brines may be divided into two general : those which produce gaseous fermentation and those which form or scum, on the surface of the brine when exposed to air but when ted from direct sunlight.

hells, Bell, and Jones (1953) described pictorially the morphology gmentation of certain yeasts isolated from brines and the cucumber in an article containing 15 natural-color plates and 109 black-and- photographs. About 30 species and strains of yeasts belonging to era are shown in this unusually fine publication.

*surface Brine Yeasts.* Basic studies relating to the identity of found in the brines of cucumbers have been carried out in the ern brining area by Etchells and Bell (1950) and in the Northern g area by Etchells, Costilow, and Bell (1952). Some of the results ir findings are summarized in Table 103. It will be noted that *psis caroliniana* (*lactis-condensi*) and *Brettanomyces versatilis* minated in the brines of eastern North Carolina, whereas *B. versatilis*, *spora rosei*, *Torulopsis holmii*, and *Hansenula subpelliculosa* were lent in the brines of the Northern area. The main difference between

TABLE 103. PATTERN OF PRINCIPAL SPECIES OF BRINE YEASTS FOUND IN COMM
CUCUMBER FERMENTATIONS

| Genera and species found in brines | Northern brines * | | Southern brine | |
|---|---|---|---|---|
| | No. of isolates | Per cent isolates | No. of isolates | Pe isc |
| *Brettanomyces* ‡ | 132 | 29.2 | 588 | 4 |
| *B. versatilis* | 103 | 22.8 | 561 | 3 |
| *B. sphaericus* | .... | .... | 27 | |
| *B. sphaericus* var. | 29 | 6.4 | | |
| *Torulopsis* | 103 | 22.8 | 721 | 4 |
| *T. holmii* | 67 | 14.8 | 4 | |
| *T. caroliniana (lactis-condensi)* | 36 | 8.0 | 717 | 4 |
| *Torulaspora* | 68 | 15.0 | 6 | |
| *T. rosei* | 68 | 15.0 | 6 | |
| *Hansenula* | 59 | 13.1 | 49 | 3 |
| *H. subpelliculosa* | 59 | 13.1 | 49 | 3 |
| *Saccharomyces* § | 46 | 10.2 | 59 | 4 |
| *S. halomembranis* | 28 | 6.2 | | |
| *S. globiformis* | 14 | 3.1 | | |
| *S. species A* | 3 | 0.7 | | |
| *S. pastori* | 1 | 0.2 | | |
| *Saccharomyces* | 22 | 4.9 | | |
| *S. globosus* | 22 | 4.9 | | |
| *Candida* | 7 | 1.5 | | |
| *C. krusei* | 7 | 1.5 | | |
| *Debaryomyces* | 5 | 1.1 | | |
| *D. membranaefaciens* var. *Hollandicus* | 5 | 1.1 | | |
| *Kloeckera* | .... | .... | 1 | 0. |
| Unclassified | 10 | 2.2 | 20 | 1. |
| Total | 452 | 100.0 | 1,444 | 100. |

---

* Data compiled from study by J. L. Etchells, R. N. Costilow, and T. A. *Farlowia,* 4(2): 249–264 (1952). Yeast isolates were made from 155 vat brines 22 individual brining stations in Indiana, Michigan, and Wisconsin during the 1950 seasons. The brines contained 4–18 per cent by weight of salt and 0.27- per cent acid (as lactic) and indicated pH levels of 4.8–3.1. Most of the isolates obtained during a fermentation period of 2–105 days; however, some were from brines 12–14 months old.

† Data compiled from study by J. L. Etchells and T. A. Bell, *Farlowia,* 87–112 (1950). Yeast isolates were made from 42 vat brines at two commercial in eastern North Carolina during the 1946 and 1947 seasons. Brine strengths from 10 to 18 per cent by weight.

‡ Lodder and Kreger-van Rij consider this to be *Torulopsis.*

§ Reported as *Zygosaccharomyces* sp.

ɪra of the two areas was the occurrence of *Saccharomyces globosus* northern brines. *T. holmii* predominated during the 2d to 30th days fermentation, while *B. versatilis* appeared to be most prevalent the period between the 70th and 110th days of the fermentation of ern brines.

*n-forming Brine Yeasts.* Studies concerning the types of yeasts ısible for the formation of wrinkled films on the surfaces of brines ›d to the air and sheltered from the direct rays of the sun have been ed by Etchells and Bell (1950) and by Etchells, Costilow, and 1950).

ısts associated with film formation (during the 1947 and 1948 sea- in 40 commercial cucumber brines containing 5 to 14 per cent by t of salt, which were found in vats located in North Carolina, ːia, Michigan, Indiana, and Wisconsin, were isolated and identified chells and Bell. *Debaryomyces membranaefaciens* var. *Hollandicus* he predominating species. Other yeasts included *Endomycopsis* *ri, S.* (*Zygosaccharomyces*) *halomembranis* nov. sp., and *Candida* *i*. Yeasts of the genus *Debaryomyces*, which were found in all brines, ssed high salt tolerance and the ability to utilize a large number of ınces as carbon sources.

ring a study made in 1950, Etchells, Costilow, and Bell found cul- of *Pichia alcoholophila* and *H. anomala* in brines of low salt con- ation (usually less than 2 months old). These yeasts grow poorly, all, in brines containing over 10 per cent salt, according to the ːs of salt-tolerance tests.

**rbic Acid as Yeast Inhibitor.** A number of scientists have indi- that sorbic acid may be used to prevent the growth of yeasts and in cucumber fermentations.

llips and Mundt (1950) reported that 0.1 per cent sorbic acid y inhibited the surface growth of yeasts and molds in cucumber ntations, inhibited lactic acid production during the early period of ntation but not during the later part, and did not adversely affect avor of the finished pickle. Their observations with respect to the itory effect of sorbic acid were confirmed by Jones and Harper ).

ıard and Vaughn (1952) showed that a 0.12 per cent concentration of acid exerted a markedly inhibitory effect on catalase-positive omycetes, bacteria, molds, and yeasts when the initial pH levels of nedia were in the range of 5 to 5.5 and phosphate salts were present hat it permitted growth of lactic acid bacteria and clostridia.

eneman and Costilow (1955) reported that 0.1 per cent sorbic acid ined with 0.5 per cent acetic acid prevented the growth of three ns of yeasts from spoiled pickles and *L. plantarum*. This combina- of acids was effective in inhibiting these microbes in sucrose solu- of 2 to 40 per cent concentrations. When 0.05 per cent of sorbic was used, 1 per cent of acetic acid was required in 30 to 40 per cent se solutions and 2 per cent acetic acid in 2, 20, and 30 per cent se solutions to inhibit the osmophilic yeasts which were tested.

Costilow, Ferguson, and Ray (1955) studied the effect of sorb
on the microorganisms associated with cucumber fermentations.
found that it inhibited the species of yeasts common to these fer
tions very effectively at pH 5 and below. All the yeasts tested (*B.*
*tilis, H. subpelliculosa, T. rosei, T. caroliniana, T. holmii, S. hal*
*branis, C. krusei, D. membranaefaciens* var. *Hollandicus, Endom*
*ohmeri,* and a species of *Rhodotorula*) were inhibited at a pH of 4
salt-free medium by 0.04 per cent sorbic acid, in 4 per cent salt br
0.02 per cent sorbic acid, and in 8 per cent salt broth by 0.01 pe
sorbic acid. *L. plantarum, P. cerevisiae,* and *L. brevis* were not a
greatly by the presence of sorbic acid at different pH levels.

Costilow, Robbins, and Coughlin (1956) determined the effect of
acid (or sodium sorbate) on yeast and lactic acid fermentations in
cucumbers containing 5.3 to 10.6 per cent salt. No appreciable pellic
mation or fermentation was observed in lots containing a minim
0.025 per cent sorbic acid. The development of yeasts was greatly de
by 0.01 per cent sorbic acid but was not prevented over an ext
period. The production of acid normal to the fermentations was no
nificantly affected by 0.01 to 0.025 per cent sorbic acid (added as so
sorbate); however, variable results were obtained at a 0.05 per cent
The use of 0.1 per cent sorbic acid inhibited acid formation and re
in a lower maximum acidity.

Costilow et al. (1957) observed that no significant yeast fermenta
developed in cucumber brines treated with sorbic acid of 0.025 to 0.
cent concentration. However, acid formation was markedly inhibit
0.1 per cent sorbic acid, also by 0.05 per cent sorbic acid in the ca
fermentations brined at 40° salinometer.

Later studies by Costilow (1957) revealed that the use of sorbic
in cucumber brines greatly reduced the occurrence of bloater-type
age. The use of sorbic acid in concentrations of 0.02 to 0.10 per cent
duced no significant differences in the control of bloater formation
in a concentration of 0.01 per cent the acid was not as effective.
levels of sorbic acid also delayed the curing process, particularly
the cucumbers were brined at a high salt concentration or when the
perature of fermentation was lower.

**Control of Scum.** Scum formation is caused commonly by
forming yeasts (mycoderma), although molds may occasionally appe
scum on the surface of brine unless proper care is exercised. The o
isms in the scum utilize the acid produced by the acid-formers
sugars, produce off-odors, and result in the production of inferior prod

Vats are usually erected outdoors, since scums are less likely to
when the surface of the brine is exposed to the action of sunlight.
ever, such a location is not always advantageous, for rain may dilut
brine and dust may be a problem.

Some investigators have endeavored to control scum formation thr
the use of ultraviolet light. Rahn (1931) found that the irradiati
the brine for 30 min. each day with ultraviolet light (from a
lamp) at a distance of 6 ft. from the surface prevented the formati

Fabian and Bryan (1932) used a Cooper-Hewitt mercury-vapor olet lamp to irradiate mycoderma. They found that there was rable variation in resistance to ultraviolet light exhibited by differ- ains of mycoderma, that young cultures were more resistant than s, and that it was difficult to destroy mycoderma when the scum ick.

ells and Veldhuis (1939) attempted to control the growth of myco- by the use of mineral oil. They discovered that the culture of erma used by them was able to grow under a layer of the oil and l acid and dextrose from the brine. However, it appeared that the a light mineral oil resulted in a somewhat slower rate of the disap- ce of the brine acid.

ian (1940) has demonstrated that scum formation on the surface of e dill-pickle brine may be prevented by the addition of a few drops stard oil or by covering the surface with paper or some other suit- aterial that has been previously impregnated with mustard oil. Blum and Fabian (1943) studied the effect of 32 spice essential oils ven components on the control of microbial surface growth. They that oil of mustard was superior to the rest, after which followed non, cassia, and cloves.

use of plastic covers has already been referred to.

**cessing.** The amount of salt removed depends upon the use that e made of the pickles. Unless they are used as salt pickles, a large out not all, of the salt is removed.

salt pickles are placed in tanks equipped with a supply of steam. mperature is raised to 120°F. (49°C.), or above, and the pickles d by means of paddles or compressed air. The water may be d and the process repeated until the desired salt content is ed.

**ding.** Pickles are sorted in order to meet the varied demands of nsumer. They may be graded for shape and size. Straight pickles ute the highest grades; the nubs and crooked ones constitute a grade; while broken and poorly shaped pickles may be used in the tion of sweet relish or chowchow.

les 2 to 3 in. in length are classified as small pickles and are used tling and sweet pickles. Medium-sized pickles, those 3 to 4 in. long, d in making dill or sweet pickles, while large pickles, 4 in. or more th, are used principally for sour pickles.

**paration of Pickles.** Although there is a large number of pickle ts, only a few of them will be discussed here. The interested reader erred for further details to the publications listed at the end of apter or to works on food technology.

*pickles*, "immature cucumbers preserved in a solution of common ith or without spices,"[1] may be converted into sour pickles, sweet s, mixed pickles, or other pickle products.

---

. *Dept. Agr., Service and Regulatory Announcements, Food Drug Admin.*, Revision 5, Nov. 5, 1936.

*Sour pickles* are made from salt pickles that have been proc drained, and covered with vinegar. A 45- to 50-grain vinegar, di vinegar being preferred usually, is added to cover the pickles. very sour pickles are desired, it is customary to add a 40- to 45- vinegar first and after a few days to replace it with the desired str of vinegar, which may be as high as 60 to 75 grains.

*Genuine dill pickles* possess a fine flavor that is the result of aging of biochemical changes brought about by microorganisms (princi bacteria) during fermentation, as well as the use of dill leaves (*Anet graveolens*) to give the characteristic flavor.

The method for preparing dill pickles differs from that of prepa salt pickles in two important respects: a weaker brine is used, and s are added to the cucumbers. The spices do not interfere with the n fermentation, but the salt concentration of the brine is low enoug permit the growth of undesirable microorganisms; hence special pre tions should be observed in the manufacture of dill pickles.

Dill pickles are usually prepared in barrels that have been caref cleaned and sometimes paraffined. Part of the dill (green, dry, or bri and the other spices are placed at the bottom of the barrel. Cucum are added and the rest of the spices distributed evenly at the middle top of the barrel. The barrel may or may not be headed, brine is ad and the barrel incubated at a temperature of around 70°F. (21.1°C.)

The addition of sugar, 2 lb. per barrel, favors the early developm of bacteria. Sufficient vinegar or acetic acid to bring the pH to 4. desirable, while the addition of a starter is advocated.

One formula for dill pickles calls for the use of 1 lb. of the follow mixture in a 45-gal. barrel: 4 lb. of whole allspice, 2 lb. of coriande mustard seed, 2 lb. of black pepper, and 1 lb. of bay leaves. From 8 lb. of green or brined dill, or 3 to 4 lb. of dried dill, is added to same barrel, while a 7 to 8 per cent brine is commonly used.

In the dill-pickle fermentation, Gram-positive cocci predomin (Fabian and Wickerham, 1935) at the beginning. Short rods, most which are Gram-positive, replace the cocci. Toward the end of the mentation, long rods predominate, most of these being Gram-positive. approximately 8 to 10 days after the start of the fermentation the stro acid-producing bacteria are at a maximum.

*Imitation dill pickles* may be prepared from salt pickles that have b processed, soured, drained, and placed in tight containers with dill, ot spices, vinegar, and brine. The acid of imitation dill pickles is princip acetic acid, while in genuine dill pickles the acid is mainly lactic acid.

*Sweet pickles* are "pickled cucumbers or other vegetables sweete with sugar and/or dextrose."[1] They may be prepared from salt pic that have been soured and drained. A sirup is made by dissolving 10 lb. of sugar in 1 gal. of vinegar. The pickles are placed in a contai one-half their weight of sirup is poured over them, and spices are usu

---

[1] *U.S. Dept. Agr., Service and Regulatory Announcements, Food Drug Adm* No. 2, Revision 5, Nov. 5, 1936.

At the end of 2 to 4 months, the sugar is largely inverted and the
have become darker green in color.

lsworth and Fabian (1939) have discussed the use of lactic acid in
nation with acetic acid in the finishing of sweet pickles, processed
kles, and pickle products. Ratios of lactic acid to acetic acid of
.4 per cent of lactic acid, as acetic acid, to 1.6 per cent acetic acid),
produced the best results.

influence of sugar and acid on flavors in pickles was determined by
l of about 200 people in a study made by Switzer and Fabian
. There was a slight preference for a combination of 75 per cent
e and 25 per cent dextrose, rather than for sucrose alone, in a
sirup of 20-grain acidity. The tasters in general were more sensi-
slight increases in the acidity than they were to slight decreases in
eetness.

*ed pickles* consist of two or more vegetables in the same container—
bers, cauliflower, green peppers, onions, beans, or green tomatoes
common vegetables used in the manufacture of mixed pickles.

*her dills* are dill pickles containing garlic as an important spice.

*ad-and-butter Pickles.* Cucumbers of 1,200 to 1,800 count (Etchells
hmer, 1941) (and usually onions) are soaked in potable water,
d in a rotary washer, and sliced. The slices are placed in a tank
as been thoroughly cleaned, covered with a brine of 25 to 30°
meter, and permitted to stand overnight (about 12 hr.). The slices
aced in 12.5- or 25-oz. or other types of glass jars, each containing
rd and celery seed. A hot (160 to 170°F.) spiced sirup is poured
he slices and the glass jars are sealed. The pickles are pasteurized
5°F. for 15 min. or at 160°F. for 20 min. and then cooled promptly.
teurization is essential since acid-formers and yeasts develop during
ine treatment and may survive the application of the hot liquor,
ausing spoilage of the pickles. The sporeformers, which originate
he cucumbers or from the adherent soil, survive pasteurization but
little or no increase during storage and are not therefore a source
ilage. Moldy cucumbers, regardless of the degree, should not be
or making fresh cucumber pickles, since the mold produces off-
s.

**steurization of Pickles.** Pickles are pasteurized to prevent unde-
e changes in texture and flavor and spoilage from taking place.
ver, it is not essential to pasteurize all types of pickles. Salt stock,
ample, may keep satisfactorily for several years if the brine concen-
n is maintained at a sufficiently high level, usually at 60 to 70° sali-
ter. Sour pickles keep on account of their vinegar content, and
pickles keep because of their combined vinegar and sugar.

the other hand, there are a number of types of pickles that require
urization. These include pickles made from fresh or partially fer-
d cucumbers and certain of the dill pickles, especially those only
lly fermented. Pasteurization is also beneficial in the case of gen-
dill pickles, for it aids in the retention of their original firmness even
storage for several months.

Pasteurization destroys spoilage types of microorganisms, thus a ing fermentation or preventing its continuation. It destroys the formers, which are responsible for the normal fermentation of brin which may cause spoilage in stored-fresh or partially fermented pi and the yeasts, which cause vigorous gas production. Such gas favo production of bloaters, the breakage of glass jars, or the bursti sealed cans.

Pickles should be pasteurized in such fashion that the temperatu the center of every glass jar or can reaches 165°F. and is held ther 15 min., followed by prompt cooling to 100°F. or below. An alte method is to pasteurize at 160°F. for 20 min., followed by prompt co Details of the pasteurization procedure are given by Etchells and (1942, 1944).

**Spoilage of Pickles.** Microorganisms and their products are the cipal causes of the spoilage of pickles. Some of the bacteria that about a normal fermentation during brining, such as the lactic-a formers, may cause subsequent spoilage of the pickles during stora not destroyed. As a matter of fact, the acid-formers and yeasts are an the chief causes of spoilage of pickles (Etchells, 1938; Etchells Goresline, 1940; Etchells and Ohmer, 1941). Both yeasts and a formers may survive improper pasteurization. Molds metabolize lactic acid produced during the curing process and may produce cha in the texture as well as the flavor of pickles as a result of the actio their enzymes and the end products formed by them.

*Bloater Formation.* Bloaters are cucumbers which have been defor or misshaped through the action of gases produced within them. types, "balloon" and "lens," are common. In the former type, the car of the cucumbers are separated by the pressure of the gas and forced against the skin, giving rise to a large cavity which is filled with ga liquid. In the latter type, the individual gas pocket has the general sh of a lens and is smaller in size than the balloon type. The lens-sha spoilage occurs principally in small cucumbers.

Bloaters are caused by the gaseous fermentation of microorganis particularly yeasts. The relationship between the initial salt concen tion and the percentage of bloaters formed during the curing of salt st has already been mentioned (page 346). Species of the genus *Aeroba* may cause bloaters under some conditions. Etchells and Bell (19 observed serious bloater damage in two commercial-scale experim concerned with the control of bloaters in dill pickles of the overn variety contained in 50-gal. barrels with 3 to 4.5 per cent salt. The gro of gas-forming yeasts had been controlled by the use of 0.05 and per cent sorbic acid or its sodium salt. Large numbers of acid-produ bacteria, such as *L. plantarum, L. brevis,* and *P. cerevisiae,* were fou with a high proportion identified as *L. brevis,* a gas-former. Isolate these three bacterial species from 36 barrels of overnight pickles tested for their ability to produce bloater formation in quart jars of teurized cucumbers equalized at 4 to 5 per cent salt and 0.25 per lactic acid and at a pH of 4. Bloaters were formed in all jars inocul

*L. brevis,* but none in jars inoculated with *L. plantarum* or *P. siae.*

se findings indicate that microbes other than yeasts may have to be ed in cucumber fermentations of low salt concentration in order to nt bloater formation.

*ening.* The *softening* of cucumber salt stock is a serious cause of o the pickle industry. The annual loss has been estimated at 00 to $750,000 (Etchells and Jones, 1951).

ening is due to a pectin-splitting enzyme, which is similar to poly- uronase, an enzyme concerned with the hydrolysis of the glycosidic which occur between deesterified galacturonide residues in pectic ials (McCready and Owens, 1954). A second pectin-splitting ne is known as "pectinesterase" (this is concerned with the hydroly- the ester bonds of pectic materials and yields methyl alcohol and acid).

ymes capable of producing pectin splitting have been elaborated rtain species of yeasts, molds, and bacteria and have been found ated with the cucumber. It has not been possible, up to the present to implicate any one group of microorganisms with the softening of ock.

hells, Jones, and Bell (1952) screened 143 subsurface and film yeasts effort to determine their ability to produce the salt-stock-softening ne, but none was found to be a potential source of the enzyme. Most film-forming yeasts were able to convert pectin to pectic acid. yeasts (not isolated from cucumber brines) were able to produce oftening enzyme.

rtje and Vaughn (1953) studied the pectolytic activity of species of nus *Bacillus.* Cultures of *B. subtilis* and *B. pumilis* elaborated ing enzymes under certain conditions. The crude enzyme prepara- of these species were able to soften cucumbers and olives only when I of the reaction mixture was 5.7, or higher. The authors concluded hese bacteria are not involved in the softening of cucumbers or which undergo a normal acid fermentation.

ughn et al. (1954) tested soils (used for cultivation of cucumbers); bers, cucumber blossoms and leaves; the shavings from fermenta- anks; water; salt; brine; and spices for the presence of micro- isms able to soften a polypectate gel medium. Aerobic, sporeform- acteria of the genus *Bacillus* predominated. There were 43 isolates *subtilis,* 2 of *B. subtilis* var. *alterimus,* 21 of *B. pumilis,* and 2 of *B. osporus.* They found that the bacilli could utilize lactic acid and te the pH of the brines, provided that the initial values were not tory. Cultures of *Fusarium solani, Aspergillus,* and *Penicillium* able ften the polypectate gel were also isolated. All the microbes were o grow in the presence of 6 per cent sodium chloride.

ughn and his associates concluded that species of the genus *Bacillus* cause the softening of cucumber salt stock if (1) the bacilli pre- nated the microbial population of the cucumbers and their brines, he pH of the brined cucumbers was in the desired range for softening,

probably 5.5, or above, and (3) the desirable lactic acid fermenta
was retarded or arrested in some manner such that the pH levels of
brine cucumbers remained relatively high for a number of days.

An enzyme similar to the pectinolytic enzyme has been found by
(1951) to be associated with dry cucumber seeds, staminate and pollina
pistillate flowers, and ripe cucumbers.

A cellulolytic ($C_x$) enzyme has also been implicated in the softenin
cucumbers. Data on the occurrence of this enzyme in cucumber bri
have been presented by Etchells, Bell, and Jones (1955).

A method for testing cucumber salt-stock brines for both the pect
olytic and the cellulolytic enzymes has been described by Bell, Etche
and Jones (1955). The procedure is similar for each enzyme; howev
sodium pectate is used for the determination of the pectin-splitt
enzyme and sodium carboxymethylcellulose for the cellulolytic enzy

Research carried out by Etchells, Bell, and Jones (1955) has dem
strated that the pectinolytic and cellulose-decomposing enzymes secre
by molds are the principal cause of the softening of cucumbers dur
brine curing. The molds concerned, chiefly species of the genera *Fu*
*rium, Penicillium, Phomaceae, Cladosporium, Alternaria, Dematiace*
*Mucor, Trichoderma, Myrothecium,* and *Aspergillus,* secrete their
zymes into the cucumber flowers, which eventually may become dri
The introduction of the fresh or dried flowers containing the softeni
enzymes, along with the cucumbers to which they are attached, suppl
the brining tanks with the spoilage factors.

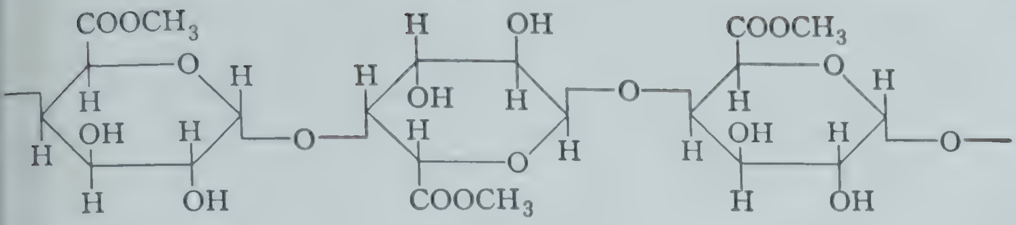
It has been observed that cucumbers harvested early in the seas
retain several times as many blossoms as those harvested later. Flow
retention is also greater on smaller cucumbers. Brine tanks filled wi
small cucumbers containing a high percentage of flowers or with expe
mentally added flowers possessed high enzyme activity, and the salt sto
was usually soft or inferior in firmness.

Because of this basic knowledge, it is now possible to reduce loss
caused by softening. One of the most effective procedures is to drain
the original cover brine about 36 hr. after the vats are filled, in order
reduce the amount of softening enzyme. New brine is added to repla
that drained off. Removal of the blossoms and thorough washing of t
cucumbers helps also. When in doubt about the quality of the brine,
simple test may be carried out to indicate whether or not enzymes whi
cause softening of cucumbers are present.

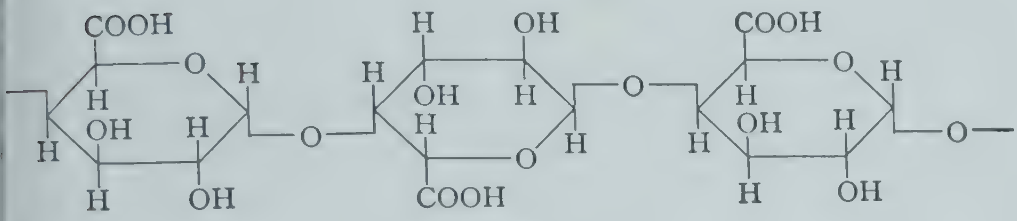
DECOMPOSITION OF PECTIN AND PECTIC ACID. A scheme for the decom
position of pectin and pectic acid, the formulas of which are given belo
has been devised by Demain and Phaff (1957) and is shown in F
59.

*Shriveling* is usually caused by placing the cucumber or pickle in t
strong a salt, sugar, or vinegar solution.

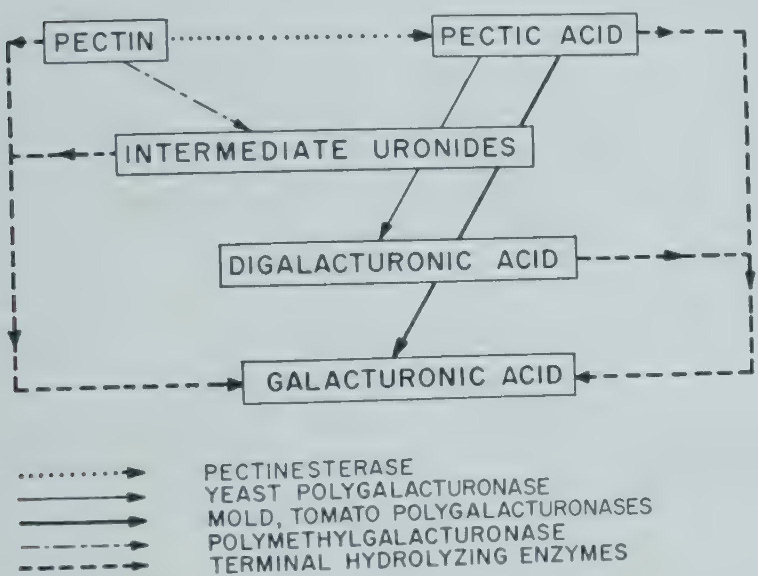
*Pickle blackening* may be caused by *B. nigrificans*, a strain of *B. mese*
*tericus*. The presence of a carbohydrate, such as dextrose, the absence
adequate quantities of nitrogenous substances, and a reaction that
practically neutral or slightly alkaline favor blackening (Fabian a


COOCH$_3$
O
H
OH
H
OH
H
H
OH
H
O
COOCH$_3$

Pectin


COOH
O
H
OH
H
OH
H
H
OH
H
O
COOH

Pectic Acid


PECTIN
PECTIC ACID
INTERMEDIATE URONIDES
DIGALACTURONIC ACID
GALACTURONIC ACID
PECTINESTERASE
YEAST POLYGALACTURONASE
MOLD, TOMATO POLYGALACTURONASES
POLYMETHYLGALACTURONASE
TERMINAL HYDROLYZING ENZYMES

9. Scheme of pectin and pectic acid decomposition by the pectic enzymes. *esy of A. L. Demain and H. J. Phaff, Jour. Agr. Food Chem.,* **5**: 60 (1957). *ight* 1957 *by the American Chemical Society and reprinted by permission of* *pyright owner.*]

uis, 1934). A water-soluble pigment produces the darkening of the and pickles.

e production of iron sulfide is also a cause of pickle blackening. rogen sulfide ($H_2S$) may be produced by bacteria or by chemical s. In the presence of iron salts, the sulfide of iron is produced. Iron le is formed at a neutral or slightly alkaline reaction (Fabian, Bryan, Etchells, 1932).

e prevention of spoilage may be accomplished by avoiding the itions that lead to it. The adequate washing of cucumbers, the use roperly cleaned and sanitized vats, the employment of the most ble initial concentration of brine, and the use of adequate pasteuri- n are some of the general measures which may be used to reduce or ent spoilage.

**Preservation-prediction Chart.** A prediction chart has been de
by Bell and Etchells (1952) which, according to the authors, should
to reduce spoilage, save sugar, and standardize sweetening formulas.
chart was developed through studies of 35 cultures of yeasts respon
for a gaseous type of spoilage of sweet cucumber pickles. The yeasts
closely related to *S.* (*Z.*) *globiformis.*

The preservation-prediction chart is shown in Fig. 60. Three lines
given in the chart which represent (*A*) sucrose plus acetic acid, (*B*)

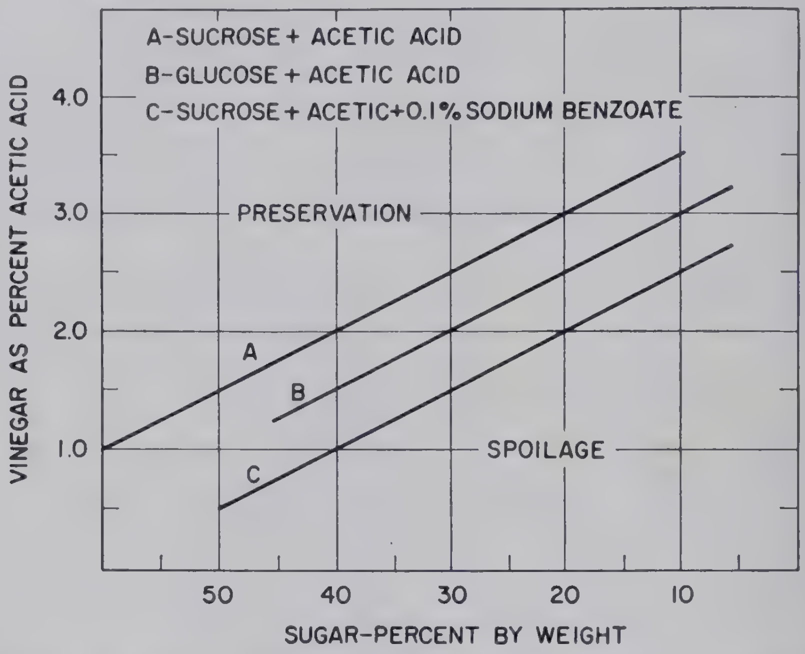


Fig. 60. Preservation-prediction chart; the area above each line represents preser
tion for that particular combination of acid, sugar, and/or sodium benzoate; the a
below each line represents the spoilage zone. [*Courtesy of T. A. Bell and J.*
*Etchells, Food Technol.*, **6**(12): 468 (1952).]

cose plus acetic acid, and (*C*) sucrose plus acetic acid plus 0.1 per c
sodium benzoate. The areas above the lines represent preservation; th
below the lines, spoilage.

Mathematical formulas for the three lines may be applied to calcul
the amounts of sugar, acid, or benzoate required to prevent gaseous f
mentation. These follow:

(*A*) Sucrose + acetic acid:

$$S_s = 80 - (20 \times A), \text{ or}$$

$$A = \frac{80 - S_s}{20}$$

(*B*) Glucose + acetic acid:

$$S_g = 70 - (20 \times A), \text{ or}$$

$$A = \frac{70 - S_g}{20}$$

Sucrose + acetic acid + benzoate:

$$S_s + B = 60 - (20 \times A), \text{ or}$$

$$A + B = \frac{60 - S_s}{20}$$

ese formulas, $A$ = per cent acetic acid, $S_s$ = per cent sucrose by t, $S_g$ = per cent glucose by weight, and $B$ = 0.1 per cent sodium ate.

**crobiological Examination of Pickles.** Etchells and Jones (1946) described tentative methods for the microbiological examination of l, salted, and pickled vegetables and vegetable products. The report its directions for the collection, storage, preparation, and micro- examination of the samples and for the preparation and use of ntial media.

pickles and pickle products listed in the following classification, luced from the article by Etchells and Jones, are considered in the presented in Table 104.

icumber Pickles and Similar Pickle Products
- *Salt Stock for Cured Pickle Products*
  1. Cucumbers (and onions, peppers, tomatoes, cauliflower, melon rinds, etc.)
  2. Genuine dill pickles (from cucumbers or tomatoes)
- *Finished Pickle Products from Brine-cured Stock*
  1. Sweets
  2. Sours
  3. Mixed
  4. Relishes
  5. Artificial or processed dills
- *Types of Pasteurized Pickles (Not Brine-cured)*
  1. Dills (sliced or whole)
  2. Sweets (sliced or whole)
  3. Relishes (mixed vegetable)
  4. Vegetables other than cucumbers (onions, peppers, tomatoes, etc.)

ined and Salted Vegetables for Non-pickle Use
- *Brined*
  1. Okra (whole)
  2. Celery (whole)
  3. Sweet pepper hulls
- *Dry-salted*
  1. Corn
  2. Lima beans
  3. Peas
  4. Snap beans
  5. Okra (cut)
  6. Celery (cut)

**e Salinometer.** The salinometer (or salometer) is an instrument s very useful in measuring the salt concentration of a brine. The on the salinometer is divided into 100 degrees, the zero graduation ting the reading of the instrument when placed in pure water at and the 100° graduation indicating the reading obtained in a satu- solution of sodium chloride at 60°F. (26.5 per cent salt). Each represents slightly more than ¼ per cent (0.265 per cent) of salt.

TABLE 104. GUIDE TO THE BACTERIOLOGICAL EXAMINATION OF CERTAIN BRINED, SALTED, AND PICKLED VEGETABLES AND VEGETABLE PRODUCTS *

| Microbial group involved | Culture medium used (and abbreviation) | Classes of fermenting products in which microbial group is likely to be present † | Remarks concerning microbial groups |
|---|---|---|---|
| Coliform bacteria | Brilliant green lactose bile agar (BG) or violet-red bile agar | I*A*: Fermenting salt stock vegetables and genuine dills<br>II*A* and *B*: Brined and salted vegetables for nonpickle use | Gaseous fermentation. Group salt-tolerant but not acid-tolerant. Most likely absent from finished pickles owing to acid content; same is true for brines when appreciable acid is present |
| Acid-forming bacteria | Nutritive caseinate agar ‡ (NC) | I*A*: Fermenting salt stock vegetables and genuine dills<br>I*B*: Finished pickle products<br>I*C*: Pasteurized pickle products | Acid fermentation. Salt-tolerant up to 15 per cent; not likely to be found in brined and salted vegetables above this concentration (II*A* and *B*) |
| Salt-tolerant cocci | Nutritive caseinate agar | II*A* and *B*: Brined and salted vegetables for nonpickle use; also, other high-salt vegetables without appreciable acidity | No outstanding characteristics of fermentation reported. Group salt-tolerant but sensitive to acid. Can grow at refrigerator temperature (1.7°C.) at approximately 10 per cent salt |
| Yeasts, mycoderma, and molds § | Acidified dextrose agar (AD) | All classes of products (I*A*, *B*, and *C*; and II*A* and *B*) for yeasts. Molds and mycoderma on liquid surface of products exposed to air and sheltered from sunlight | Yeasts: gaseous fermentation; acid- and salt-tolerant. Molds and mycoderma; acid- and salt-tolerant; both groups utilize acid of products and require free oxygen for growth |
| Obligate halophiles | Liver broth plus salt (LBS) | II*A* and *B*: Brined and salted vegetables for nonpickle use. Also, in other vegetable brines at high-salt concentration | Gaseous fermentation. Group requires about 15 per cent salt in culture medium and reduced oxygen tension. Sensitive to acid. General fermentation behavior not well known. |
| Butyric acid group | Liver broth plus particles (without salt) (LB) | Uncommon in brined and salted vegetables; examination should be made if malodorous fermentation is detected | Malodorous, gaseous fermentation. Not particularly acid- or salt-tolerant. Active fermentations rare in properly brined or salted vegetables |

---

* J. L. Etchells and I. D. Jones, *Am. Jour. Public Health*, **36**(10): 1112 (1946).

105 gives a comparison of salt percentages and the corresponding
meter readings.

BLE 105. SALT PERCENTAGES AND CORRESPONDING SALINOMETER READINGS

| solution, cent | Salinometer reading, degrees | Salt in solution, per cent | Salinometer reading, degrees |
|---|---|---|---|
| 0 | 0 | 10.07 | 38 |
| 265 | 1 | 10.6 | 40 |
| 53 | 2 | 11.13 | 42 |
| 795 | 3 | 11.66 | 44 |
| 06 | 4 | 12.19 | 46 |
| 325 | 5 | 12.72 | 48 |
| 59 | 6 | 13.25 | 50 |
| .855 | 7 | 13.78 | 52 |
| .12 | 8 | 14.31 | 54 |
| .385 | 9 | 14.84 | 56 |
| .65 | 10 | 15.37 | 58 |
| .915 | 11 | 15.9 | 60 |
| .18 | 12 | 16.43 | 62 |
| .445 | 13 | 16.96 | 64 |
| .71 | 14 | 17.49 | 66 |
| .975 | 15 | 18.02 | 68 |
| .24 | 16 | 18.55 | 70 |
| .505 | 17 | 19.08 | 72 |
| .77 | 18 | 19.61 | 74 |
| .035 | 19 | 20.14 | 76 |
| .3 | 20 | 20.67 | 78 |
| .565 | 21 | 21.2 | 80 |
| .83 | 22 | 21.73 | 82 |
| .095 | 23 | 22.26 | 84 |
| .36 | 24 | 22.79 | 86 |
| .625 | 25 | 23.32 | 88 |
| .89 | 26 | 23.85 | 90 |
| .155 | 27 | 24.38 | 92 |
| .42 | 28 | 24.91 | 94 |
| .95 | 30 | 25.44 | 96 |
| .48 | 32 | 25.97 | 98 |
| .01 | 34 | 26.5 | 100 |
| .54 | 36 | | |

## PICKLED GREEN OLIVES

iefly, the production of pickled green olives consists of selecting
ble varieties, harvesting, grading, treating with lye, washing with
r, brining, and packaging. In order to obtain a high-grade product,
necessary to use considerable care in each of the various operations.
*rieties.* Cruess (1930) lists five varieties of olives that are suitable
green pickling. These include the Sevillano, Barouni, Manzanillo,
lano, and Mission olives, the descending order of their suitability
g given.

*Harvesting.* The olives should be picked when full sized but f any pink or red color. Green to partially yellow olives are pref Olives that are straw-yellow to pink in color do not produce th pickled products.

*Grading.* The overripe, bruised, or blemished olives are remove if the olives will not be bruised in the process, they are graded; othe grading is deferred until after the fermentation.

*Lye treatment.* Olives are treated with a dilute lye (sodium or p sium hydroxide) to remove a portion but not all of the bitter prin for some bitterness is desirable for flavor.

The concentration of lye used depends upon the variety of olive treated. A 1.6 per cent lye, or one slightly less concentrated, is advo by Cruess for Sevillano and Ascolano olives, but a 1.7 to 2 per cen is advocated for Manzanillo, Barouni, and Mission olives because contain more of the bitter principle. The use of too concentrated l prolonged treatments will remove all the bitter principle and impai flavor, color, and texture.

The time required for treating the olives with lye is dependent on temperature of the process, the concentration of the alkali, the siz the olive, and the depth of penetration desired. The temperature of lye is generally 70 to 75°F. (21.1 to 23.9°C.). Lye is permitted to p trate one-half of the way to the pit in Sevillano and Ascolano olives two-thirds of the way in Manzanillo, Barouni, and Mission olives. process usually requires 4 to 6.75 hr., during which time the olives sh be stirred occasionally.

The progress of lye penetration is followed by removing olives f time to time, slicing them, placing a few drops of phenolphthalein on cut surface, and observing the pink to red color that indicates the de to which the lye has penetrated.

*Washing.* The lye-treated olives are washed to remove the alk The olives are covered with clean water, which is allowed to stand 2 or 3 min. and then drained off. This process is repeated perhaps t times, and thereafter the water is changed about once every 3 hr.

Ordinarily about 30 hr. may be required for washing, but the act time will depend on the frequency of changing the water, the size of olives, the depth of penetration of the lye in the olives, and the concent tion of the lye.

One does not expect to remove all the lye from all the olives du washing. When 75 to 80 per cent of the olives show no pink or red c with phenolphthalein when this indicator is applied to the cut surf a satisfactory removal of lye is believed to have taken place.

*Fermentation.* For brining and fermentation the olives should placed in oak barrels. A 44° salinometer (11.66 per cent salt) brin added at once to Manzanillo, Barouni, or Mission olives. Sevillano Ascolano olives will shrivel if a concentrated brine is used. Hence Cru advocates adding a 20° brine to the olives and increasing the concent tion by 5° at 2-day intervals until the concentration remains fa constant at 28 to 30° salinometer. A final concentration of less than

ıeter is considered dangerous, for gas tends to form in the olives,
oilage, including softening, increases. The use of a brine more
rated than 32° produces olives that are too salty in flavor.

addition of 3 lb. of dextrose, dissolved in the brine, to a 50-gal.
increases the acidity of Manzanillo and Mission olives. Sugar
not be added to the other varieties during the fall of the year.

ers may be added along with the brine, for during the lye treat-
ı large part of the lactic acid and other bacteria is destroyed.
(1937), experimenting with a number of lactobacilli grown in
and sterilized tomato juice, advocates the addition of starters con-
pure cultures to green olives. He found *Lactobacillus pentosus*
bly a synonym of *L. plantarum*) to be most desirable, for olives
ted with this organism possessed a better flavor and higher acidity
ninoculated controls. *L. pentoaceticus* (probably a synonym of *L.*
likewise gave fairly good results. The brine from olives that are
in flavor and odor may be used as a starter. Such brine should
ı the fewest possible number of film yeast or mold spores; otherwise
may cause much more harm than good.

ification of the brine with a small amount of vinegar or lactic
ıs proved of value in improving the quality of the pickled olives.

*during Fermentation.* Barrels containing the olives are commonly
in the sun to ferment, although they may be incubated at a tem-
re of 70 to 80°F. (21.1 to 26.7°C.). Brine or salt is added several
ı week for the first 3 or 4 weeks and thereafter about once a week
intain a concentration of 28° salinometer and to keep the barrel
Unless the barrel is kept filled and sealed, film yeast and molds
velop and utilize the acid produced during fermentation. Since the
and keeping qualities of the olives depend in large part on the
of the brine, the necessity for preventing the growth of the yeast
ıd molds is evident.

ır may be added to the brine if the acidity fails to increase to 0.9 g.
ıter per 100 ml.

*kaging.* The olives are packaged in glass jars after the flesh has
"chalky-white" color, a characteristic flavor has developed, and
idity is equivalent to 0.9 g. per 100 ml., or greater. After the jars
ed with olives, water is added to rinse away any sediments. The
e, after draining, filled then with a 30° salinometer brine, and lactic
ıay be added if the original brine contained less than 0.5 g. of acid
0 ml. Sealing is effected, with or without the use of vacuum.

## Some References concerning Pickles

. A.: Proteolytic Enzyme Activity in Various Parts of the Cucumber Plant
l Fruit, *Botan. Gaz.*, 113: 216–221 (1951).
and J. L. Etchells: Sugar and Acid Tolerance of Spoilage Yeasts from Sweet-
umber Pickles, *Food Technol.*, 6: 468–472 (1952).
———, and I. D. Jones: A Method for Testing Cucumber Salt-stock Brine
Softening Activity, *U.S. Dept. Agr., Agr. Research Serv., ARS* 72–5, Septem-
, 1955.
l, R. H.: "Pickle and Sauce Making," R. H. Binsted, London, 1939.

Blum, H. B., and F. W. Fabian: The Influence of Salting upon Vitamins A and Vegetables, *Fruit Products Jour.*, **22**(9): 273–275, 283 (1943).

——— and ———: Spice Oils and Their Components for Controlling Microbial face Growth, *Fruit Products Jour.*, **22**(11): 326–329, 347 (1943).

Brown, C. W.: Notes on Brine Pickle Fermentation, *Jour. Bact.*, **1**: 104 (1916).

Campbell, C. H.: Changes That Take Place during the Salting of Pickles, *Ca* **50**: 45 (1920).

———: "Canning, Preserving, Pickling," The Canning Age Publishing Co., 1937.

Costilow, R. N.: Sorbic Acid as a Selective Agent for Cucumber Fermentat III. Evaluation of Salt Stock from Sorbic Acid Treated Cucumber Ferme tions, *Food Technol.*, **11**: 591–595 (1957).

———, F. M. Coughlin, E. K. Robbins, and W.-T. Hsu: Sorbic Acid as a Sele Agent for Cucumber Fermentations. II. Effect of Sorbic Acid on Yeast Lactic Acid Fermentations in Brined Cucumbers, *Appl. Microbiol.*, Vol. 5,

——— and F. W. Fabian: Effect of Various Microorganisms on the Vitamin Amino Acid Content of Cucumber Brines, *Appl. Microbiol.*, **1**: 326–329 (1

———, W. E. Ferguson, and S. Ray: Sorbic Acid as a Selective Agent in Cucu Fermentations. I. Effect of Sorbic Acid on Microorganisms Associated Cucumber Fermentations, *Appl. Microbiol.*, **3**: 341–345 (1955).

———, E. D. Robbins, and F. M. Coughlin: II. Effect of Sorbic Acid on Yeast and Lactic Acid Fermentations in Brined Cucumbers, *Bact. Proc.*, p. 28.

Cruess, W. V.: Pickling Green Olives, *Calif. Agr. Expt. Sta., Bull.* 498, October,

———: Use of Starters for Green Olive Fermentations, *Fruit Products Jour.*, **17** (1937).

———: "Commercial Fruit and Vegetable Products," 4th ed., McGraw-Hill B Company, Inc., New York, 1958.

———: Olive Products, *Ind. Eng. Chem.*, **33**: 300–303 (1941).

Demain, A. L., and H. Phaff: Softening of Cucumbers during Curing, *Jour. Agr. F Chem.*, **5**: 60–64 (1957).

——— and ———: Recent Advances in the Enzymatic Hydrolysis of Pectic S stances, *Wallerstein Labs. Communs.*, **20**(69): 119–140 (1957).

Emard, L. O., and R. E. Vaughn: Sensitivity of Sorbic Acid Media for Catalase Ne tive Lactic Acid Bacteria and Clostridia, *Jour. Bact.*, **63**: 487–494 (1952).

Etchells, J. L.: Rate of Heat Penetration During the Pasteurization of Cucum Pickle, *Fruit Products Jour.*, **18**(3): 68–70 (1938).

———: The Incidence of Yeast in Cucumber Fermentations, *Food Research*, 6 95–104 (1941).

———: A New Type of Gaseous Fermentation Occurring during the Salting Cucumbers, *University Microfilms, Publ.* 282, University of Michigan, Arbor, Mich., 1941, pp. 1–153.

——— and T. A. Bell: Classification of Yeasts from the Fermentation of Comm cially Brined Cucumbers, *Farlowia*, **4**(1): 87–112 (1950).

——— and ———: Film Yeasts on Commercial Cucumber Brines, *Food Techn* **4**(3): 77–83 (1950).

——— and ———: Bloater Formation by Gas-forming Lactic Acid Bacteria Cucumber Fermentations, *Bact. Proc.*, 1956, p. 28.

———, ———, and I. D. Jones: Morphology and Pigmentation of Certain Ye from Brines and the Cucumber Plant, *Farlowia*, **4**(3): 265–304 (1953).

———, ———, and ———: Studies on the Origin of Pectinolytic and Cell lytic Enzymes in Commercial Cucumber Fermentations, *Food Technol.*, 9: (1955).

———, ———, and ———: Cucumber Blossoms in Salt Stock Mean Soft Pick *Research and Farming*, **13**(1–4): 14–15 (1955).

———, R. N. Costilow, and T. A. Bell: Identification of Yeasts from Commer Cucumber Fermentations in Northern Brining Areas, *Farlowia*, **4**(2): 249– (1952).

———, F. W. Fabian, and I. D. Jones: The *Aerobacter* Fermentation of Cucum

ring Salting, *Mich. State Coll. Agr. Expt. Sta., Tech. Bull.* 200, June, 1945.
and H. E. Goresline: Methods of Examination of Fresh Cucumber Pickle,
*it Products Jour.*, **19**(11): 331–335 (1940).
and G. S. Harper: A Preliminary Study of Factors Affecting Quality of
kles in the Canadian Market, *Food Technol.*, **6**(8): 304–308 (1952).
and I. D. Jones: An Occurrence of Bloaters during the Finishing of Sweet
kles, *Fruit Products Jour.*, **20**(12): 370, 381 (1941).
and ———: Pasteurization of Pickle Products, *Fruit Products Jour.*, **21**(11):
-332 (1942).
and ———: Mortality of Microorganisms during Pasteurization of Cucumber
kle, *Food Research*, **8**(1): 33–34 (1943).
and ———: Bacteriological Changes in Cucumber Fermentation, *Food Inds.*,
2): 54–56 (1943).
and ———: Commercial Brine Preservation of Vegetables, *Fruit Products*
*ır.*, **22**(8): 242–246, 251, 253 (1943).
and ———: The Importance of Care in the Pasteurization of Pickle Products,
*nner*, **98**(9): 28, 64 (January, 1944).
and ———: Preservation of Vegetables by Salting or Brining, *U.S. Dept. Agr.,*
*mers' Bull.*, 1932 (revised June, 1944). (This bulletin contains a list of other
olications on the subjects of salting and brining.)
and ———: Procedure for Pasteurizing Pickle Products, *Glass Packer*, July,
4.
and ———: Procedure for Bacteriological Examination of Brined, Salty, and
kled Vegetables and Vegetable Products, *Am. Jour. Public Health*, **36**(10):
2–1123 (1946).
and ———: Progress in Pickle Research, I and II, *Glass Packer*, April, May,
1.
———, and T. A. Bell: Advances in Cucumber Pickling, in "Yearbook of
riculture, 1950–1951," U.S. Department of Agriculture, 1952, pp. 229–236.
———, and ———: Yeasts: Brigands in Brine, *Research and Farming, Progr.*
*pt.* 1, **10**: 3–4 (1951).
and H. B. Ohmer: A Bacteriological Study of the Manufacture of Fresh
cumber Pickle, *Fruit Products Jour.*, **20**(11): 334–337, 357 (1941).
and M. K. Veldhuis: Growth of Mycoderma Scum under Oil, *Fruit Products*
*ır.*, **18**(9): 265–267, 280 (1939).
———, and O. Veerhoff: Pasteurization of Genuine Dill Pickles, *Fruit Prod-*
*s Jour.*, **20**(10): 304 (1941).
F. W.: Some Important Considerations in Pickle Manufacture, *Fruit Prod-*
*s Jour.*, **9**: 376 (1930).
Pickle and Kraut Packers Conference, *Fruit Products Jour.*, **16**: 209, 214, 217,
(1937); **17**: 238, 270 (1938).
A New Method for the Prevention of Scum-forming Organisms, *Fruit Prod-*
*s Jour.*, **19**: 244 (1940).
Pickles-Tests Show Chemical Action of Spice Oils in Pickles, *Western Canner*
*l Packer*, November, 1942.
Spicing Pickles, *Western Canner and Packer*, December, 1942.
Wartime Vegetable Salting, *Food Inds.*, **16**(5) (1944).
and H. B. Blum: Preserving Vegetables by Salting, *Fruit Products Jour.*,
(8): 228–236 (1943).
and C. S. Bryan: An Attempt to Prevent the Formation of Mycoderma
ums on Pickle Brine by the Use of a Mercury Vapor Lamp, *Fruit Products*
*ur.*, **11**: 134 (1932).
———, and J. L. Etchells: Experimental Work on Cucumber Fermentation,
*, Mich. State Coll. Agr. Expt. Sta., Tech. Bull.* 126, November, 1932.
and V. Harris: Imitation Clove Oil Effective in Spicing Pickles, *Food Inds.*,
: 638–640 (1945).
and E. A. Johnson: Experimental Work on Cucumber Fermentation, *Mich.*
*ıte Coll. Agr. Expt. Sta., Tech. Bull.* 157, February, 1938.

———, C. F. Krehl, and N. W. Little: The Role of Spices in Pickled-food Spoil *Food Research,* **4**(3): 269–286 (1939).

——— and A. L. Nienhuis: Experimental Work on Cucumber Fermentation, VII, *Mich. State Coll. Agr. Expt. Sta., Tech. Bull.* 140, August, 1934.

——— and R. G. Switzer: Experimental Work on Processing and Finishing of Sw Pickles. III. Use of Dextrose and Sucrose in Manufacture of Sweet Pick *Proc. Inst. Food Technol.,* 1940, pp. 329–382.

——— and ———: Classification of Pickles, *Fruit Products Jour.,* **20**(5): 136- (1941).

——— and M. C. Van Wormer: Factors Influencing the Flavoring of Pickles w Whole Spices and Essential Oils, *Food Research,* **8**: 95–104 (1943).

——— and C. K. Wadsworth: The Utilization of Green Tomatoes, *Fruit Produ Jour.,* **23**(12): 361–362 (1944).

——— and ———: Salting Beets, Carrots, Corn, Green Beans and Spinach, Diamo Crystal Salt Co., St. Clair, Mich.

——— and L. J. Wickerham: Experimental Work on Cucumber Fermentation, V *Mich. State Coll. Agr. Expt. Sta., Tech. Bull.* 146, July, 1935.

Jones, A., and G. S. Harper: A Preliminary Study of Factors Affecting the Quali of Pickles on the Canadian Market, *Food Technol.,* **6**: 304–308 (1952).

Jones, I. D.: Salting of Cucumbers, *Ind. Eng. Chem.,* **32**: 858–861 (1940).

——— and J. L. Etchells: Physical and Chemical Changes in Cucumber Ferment tion, *Food Inds.,* **15**(1): 62–64 (1943).

——— and ———: Food Value of Brined Vegetables, *Research and Farming,* Octob 1945. (Refer also to *Am. Jour. Public Health,* **34**: 711–718 [1944].)

———, ———, and R. J. Monroe: Varietal Differences in Cucumbers for Picklin *Food Technol.,* **8**(9): 415–418 (1954).

———, ———, M. K. Veldhuis, and O. Veerhoff: Pasteurization of Genuine D Pickles, *Fruit Products Jour.,* **20**(10): 304–305, 316, 325 (1941).

———, M. K. Veldhuis, J. L. Etchells, and O. Veerhoff: Chemical and Bacterio logical Changes in Dill-Pickle Brines during Fermentation, *Food Research,* **5**(5) 533–547 (1940).

Jones, M. A. (ed.): The Brining and Pickling of Cucumbers and Other Vegetables : List of Publications with Abstracts, September 1938–December 1955 on Cooper tive Investigations by U.S. Food Fermentation Laboratory and North Caroli Agr. Expt. Sta., Raleigh, N.C.), March, 1956.

Kauble, H. B.: U.S. Patent 2,804,390, Aug. 27, 1957.

McCready, R. M., and H. S. Owens: Pectin—A Product of Citrus Waste, *Ec Botany,* **8**(1): 29–47 (1954).

Nagel, C. W., and R. H. Vaughn: Sterilization of Cucumbers for Studies of Microbi Spoilage, *Food Research,* **19**(6): 613–616 (1954).

Nortje, B. K., and R. H. Vaughn: The Pectolytic Activity of Species of the Gen *Bacillus:* Qualitative Studies with *Bacillus subtilis* and *Bacillus pumilis* in Rel tion to the Softening of Olives and Pickles, *Food Research,* **18**(1): 57–69 (1953

Pederson, C. S.: Pickle Making Principles, *Farm Research,* **16**(3): 4 (1950).

——— and M. N. Albury: Effect of Temperature upon Bacteriological and Chemic Changes in Fermenting Cucumbers, *N.Y. State Agr. Expt. Sta., Bull.* 744, Augus 1950.

——— and L. Ward: The Effect of Salt upon the Bacteriological and Chemic Changes in Fermenting Cucumbers, *N.Y. State Agr. Expt. Sta., Tech. Bull.* 28 July, 1949.

Phillips, G. F., and J. O. Mundt: Sorbic Acid as Inhibitor of Scum Yeast in Cucum ber Fermentations, *Food Technol.,* **4**(7): 291–293 (1950).

Ragheb, H. S., and F. W. Fabian: Addition of Sugar, Amino Acids and Corn Stee Liquor to Cucumber Fermentation, *Appl. Microbiol.,* **5**: 253–261 (1957).

Rahn, O.: *Canner,* July, 1931, pp. 42–43.

Samish, Z.: Studies on Pickling Spanish-type Green Olives, *Food Technol.,* 9(4) 173 (1955).

Sheneman, J. M., and R. N. Costilow: Sorbic Acid as a Preservative for Swe Cucumber Pickles, *Appl. Microbiol.,* **3**(3): 186–189 (1955).

r, R. G., and F. W. Fabian: 200 Tell What They Prefer in Pickle Sweeteners, *od Inds.*, 12(11): 38–40 (1940).

o, A. I.: Estudios sobre el aderezo de aceitunas verdes.—X. La población erobiana de las salmuerans de aceitunas, *Grasas y Aceites*, **4** (fasc. 4): 169–175 953).

: Fermentación Microbiana en el Aderezo de las Aceitunas Verdes, *Micro- ologia Española*, **6**(4): 291–306 (1953).

n, R. H., H. C. Douglas, and J. R. Gilliland: Production of Spanish-type Green ives, *Univ. Cal. Coll. Agr., Bull.* 678, April, 1943.

J. H. Levinson, C. W. Nagel, and P. H. Krumperman: Sources and Types of robic Microorganisms Associated with the Softening of Fermenting Cucum- rs, *Food Research*, **19**(5): 494–502 (1954).

is, M. K., and J. L. Etchells: Gaseous Products of Cucumber Pickle Fer- entations, *Food Research*, **4**(6): 621–630 (1939).

———, I. D. Jones, and O. Veerhoff: Influence of Sugar Addition to ines in Pickle Fermentation, *Food Inds.*, **13**(10): 54–56; **13**(11): 48–50 (1941).

orth, C. K., and F. W. Fabian: How to Use Lactic Acid in Finishing Pickles d Pickle Products, *Food Inds.*, **11**: 252, 324 (1939).

and ———: Salting Early June and Alaskan Peas, *Fruit Prod. Jour.*, **23**(10): 8–301, 316 (1944).

l, F. W., and F. W. Fabian: Experimental Work on Cucumber Fermentation. III. Influence of Garlic on the Softening of Genuine Kosher Dill Pickles, *Mich. ate Coll. Agr. Expt. Sta., Tech. Bull.* 199, February, 1945.

N. S., J. R. Gilliland, and R. N. Vaughn: Characteristics of Coliform Bac- ia from Olives, *Jour. Bact.*, **41**: 341–352 (1941).

ley, W. G.: Forty Years of Pickle Salting, *Food Inds.*, **11**: 486, 551 (1939).

## CHAPTER 20

# DEXTRANS

Dextrans are of particular interest because of their use as blood-pl
volume extenders. They are being used for other purposes in the me
field, in the food industries, and in other areas. They are of large int
in the field of fundamental carbohydrate research.

Several blood-plasma volume extenders have been studied and
including algin, pectin, casein, gelatin, gum arabic, polyvinylpyrroli
(PVP), dextran, and others. Of the materials studied, PVP and dex
appear to be most promising and are manufactured commercially
number of countries.

The product known as "dextran" was developed initially through
efforts of Grönwall and Ingelman (1944, 1945) in Sweden. Much dex
is now being manufactured by a method patented by them in 1948.

Clinical dextran possesses a number of advantages: it may be h
sterilized, it may be stored without refrigeration, it does not carry
virus which causes infectious hepatitis, it does not interfere with b
typing and may be administered to individuals of any blood type
viscosity and osmotic pressure are satisfactory, and it causes no sedin
tation of blood cells.

The methods now being used to manufacture dextran commercially
the results of the research of microbiologists, biochemists, chemists, ch
cal engineers, physicians, and others. Since dextran is introduced dire
into the blood stream of man, the requirements are high and met by c
ful control of all manufacturing steps. Rigid standards are maintai

**Structure of Dextrans.** Dextrans are polymers of D-glucose and
are polyglucosans. They differ in chemical and physical characteris
for example, in specific rotation, intrinsic viscosity, solubility in wa
nature of the solid product, and the proportion of the various glucopyra
sidal units present. They are made up of anhydroglucose units lin
together in various ways.

Jeanes et al. (1954) characterized and classified the dextrans f
96 strains of bacteria, representing 5 different genera. Linkages wer
three types: 1,6- or 1-glucosidically linked units (in 50 to 97 percenta
1,4-like linkages, and 1,3-like linkages. Table 106 shows a classifica
of dextrans, based on the proportions of the 1,3-like links, which w
determined by the periodate reaction analysis (Jeanes and Wilh
1950).

Certain details concerning the structure of dextrans are shown in
61 and 62.

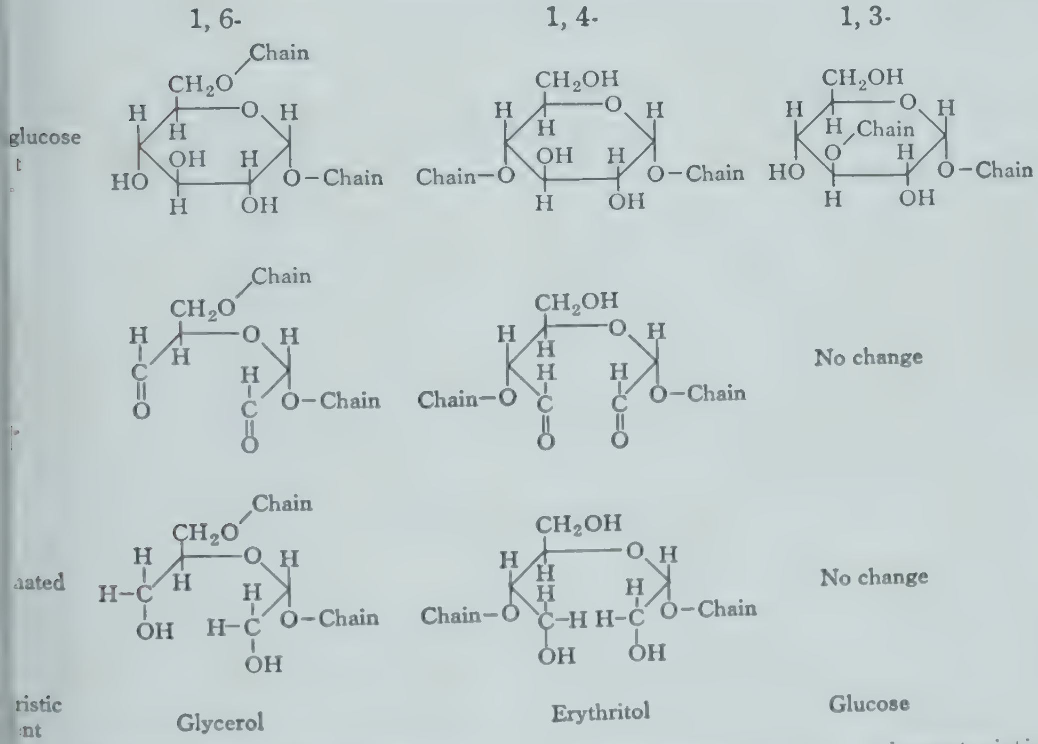
TABLE 106. CLASSIFICATION OF DEXTRANS *

| Carbon atoms of AGU involved in glucopyranosidic linkages | | | |
|---|---|---|---|
| $C_1$ (or $C_1$ and $C_6$) | $C_1$ (or $C_1$ and $C_6$) and $C_4$-like | $C_1$ (or $C_1$ and $C_6$) and $C_3$-like | No. of dextrans |
| Designation and percentage of linkages | | | |
| 1,6 | 1,4-like | 1,3-like | |
| 97–50 | 0–50 | 0–2 | 47 |
| 95–86 | 0– 8 | 3–6 | 15 |
| 85–50 | 0–36 | 6 | 28 |

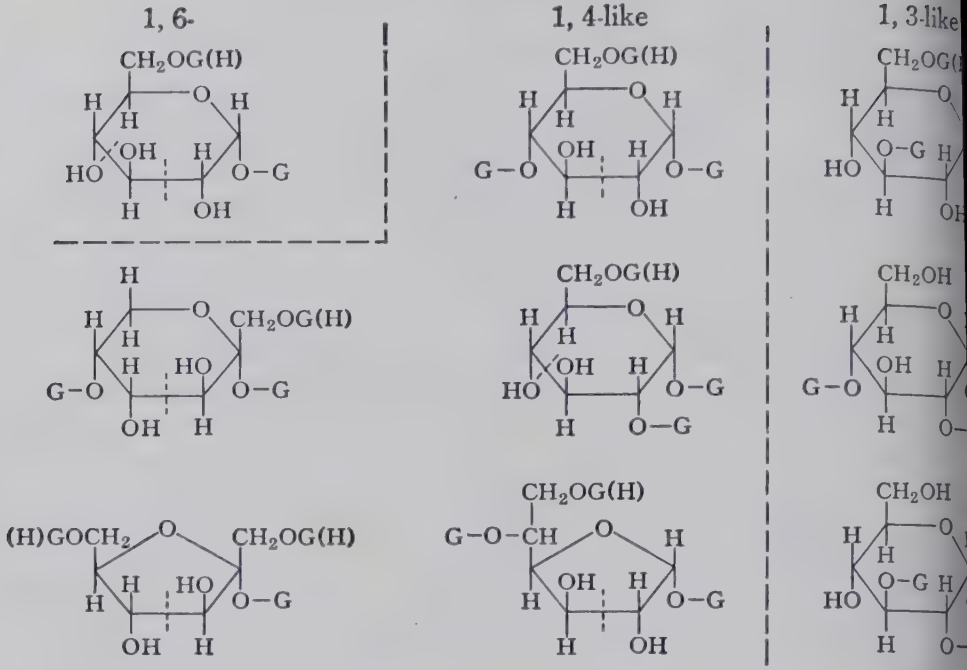
ularly heterogeneous dextrans which have been fractionated into or components belonging in two or more of the other classes ectively.

---

Jeanes et al., *Jour. Am. Chem. Soc.*, 76: 5041 (1954). Copyright 1954 by the an Chemical Society and reprinted by permission of the copyright owner.



. Degradation of periodate-oxidized anhydroglucose units to characteristic its. [*Courtesy of J. W. Sloan, B. H. Alexander, R. L. Lohmar, I. A. Wolff, and st, Jour. Am. Chem. Soc.,* 76: 4429 (1954). *Copyright* 1954 *by the American al Society and reprinted by permission of the copyright owner.*]

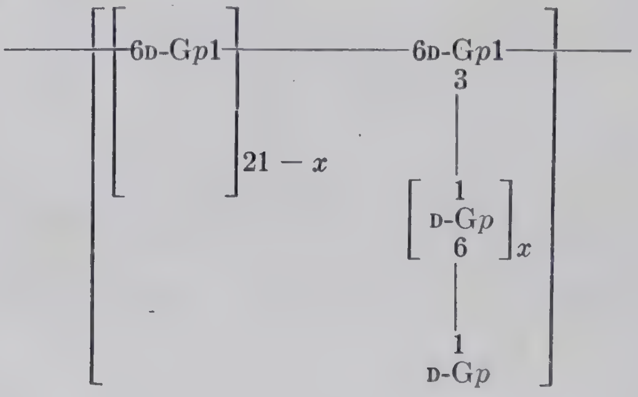


| | 1, 6- | 1, 4-like | 1, 3-like |
|---|---|---|---|
| Moles $IO_4^-$ reduced: | 2 | 1 | 0 |
| Moles HCOOH produced: | 1 | 0 | 0 |

FIG. 62. Structures representative of the three anhydrohexose types differentiate periodate oxidation: G = AGU. [*Courtesy of J. C. Rankin and A. Jeanes, Jour. Chem. Soc.*, 76: 4435 (1954). *Copyright* 1954 *by the American Chemical Society reprinted by permission of the copyright owner.*]

The dextran produced by *Leuconostoc mesenteroides* NRRL B-51 which is the organism used industrially, is a branched polysacchar which is composed entirely of anhydroglucopyranose (AG) residues ( Cleve, Schaefer, and Rist, 1956). About 91 per cent of these residues mutually joined by 1,6-linkages and about 4 per cent consist of nonred ing end groups. The remaining residues are believed to consist of 1, linked units, which represent branch points.

Based on their methylation studies, Van Cleve and associates (19 suggested that the average unit chain of NRRL B-512F dextran contai 23 anhydroglucose residues. The range of structural possibilities is sh in the formula given below, in which there are twenty-one 1,6-linked r dues, one 1,3,6-linked residue (a branch point), and one nonreducing group:



$$-\left[\left[\text{—6D-G}p\text{1—}\right]_{21-x}\text{———}\begin{array}{c}\text{6D-G}p\text{1}\\ 3\\ |\\ \left[\begin{array}{c}1\\ \text{D-G}p\\ 6\end{array}\right]_x\\ |\\ 1\\ \text{D-G}p\end{array}\right]-$$

**ve and Clinical Dextrans.** Native dextrans are those which are
y produced by fermentation or enzymatic synthesis and which
ot been altered by hydrolysis and fractionation. The weight-
molecular weight of dextran NRRL B-512F is 30 to 50 million,
etermined by light-scattering methods (Senti and Hellman, 1952;
t al., 1954). Native dextrans are modified by partial hydrolysis
ctionation to yield clinical dextrans suitable for use as the essen-
nponents in a blood volume expander. The specification of the
Forces Medical Procurement Agency (May, 1952) requires a
average molecular weight of 75,000 ± 25,000. The specification
states that the high fraction, 5 to 10 per cent, shall not exceed a
average molecular weight of 200,000 and that the low fraction,
per cent, shall not have a weight-average molecular weight less
,000. Fractions with a weight-average molecular weight of about
are preferred in clinical dextran mixtures.

**eria Producing.** Species of bacteria from a number of genera,
ng *Leuconostoc*, *Streptococcus*, *Streptobacterium*, *Acetobacter*, and
*cterium*, possess the ability to produce dextrans under certain
ons. Strains of *L. mesenteroides* are particularly useful for produc-
trans from sucrose. The properties and classification of dextrans
rains of this species have been reported by Jeanes and associates
The biochemical and cultural characteristics of the genus have
scribed by Hucker and Pederson (1930) and others.

ected bibliography on the microbiology and other aspects of dex-
s been compiled by Jeanes et al. (1952).

**um for Isolation.** McCleskey, Faville, and Barnett (1947) used
um of the following composition for the isolation of *L. mesenter-*
om sugar-cane juice at 20 to 28°C.

| | |
|---|---|
| Tryptone | 10 g. |
| Yeast extract | 5 g. |
| Sugar, raw | 100 g. |
| Agar | 20 g. |
| Water, distilled | 1,000 ml. |
| pH | 6.7 |

medium was sterilized for 15 min. at 121°C.

## PRODUCTION METHODS

rans may be produced by a fermentation process or by a process
nvolves the preparation of dextransucrase, under conditions opti-
r its elaboration, followed by enzymatic synthesis. In both proc-
fractionation procedure for separating out the clinical dextran is
ry. The enzymatic process has the advantage of producing directly
nable percentage of the dextran with a weight-average molecu-
ght in the clinical range and of being less complicated for this

## PRODUCTION OF DEXTRAN BY FERMENTATION

The industrial fermentation process described herein is that re by Bixler, Hines, McGhee, and Shurter (1953).

**Process in Brief.** *Leuconostoc mesenteroides* NRRL B-512F organism used. This coccus produces a dextran, in which the 1,6- predominate, from a nutrient solution containing sucrose, minerals, mins, and water. The native dextran formed is precipitated and sepa from the fermentation liquor. It is partially hydrolyzed with hydroc acid to yield a product with a weight-average molecular weight i clinical dextran range of 75,000 ± 25,000. Methanol is used to sep the fractions of desirable molecular weight from those with too hi too low molecular weights. The clinical fraction is purified, concent spray-dried, packed, analyzed, and finally processed for use as a volume expander or otherwise.

**Process in Detail.** A flow sheet for the commercial process is s in Fig. 63, while a floor plan is shown in Fig. 64. In order to pr

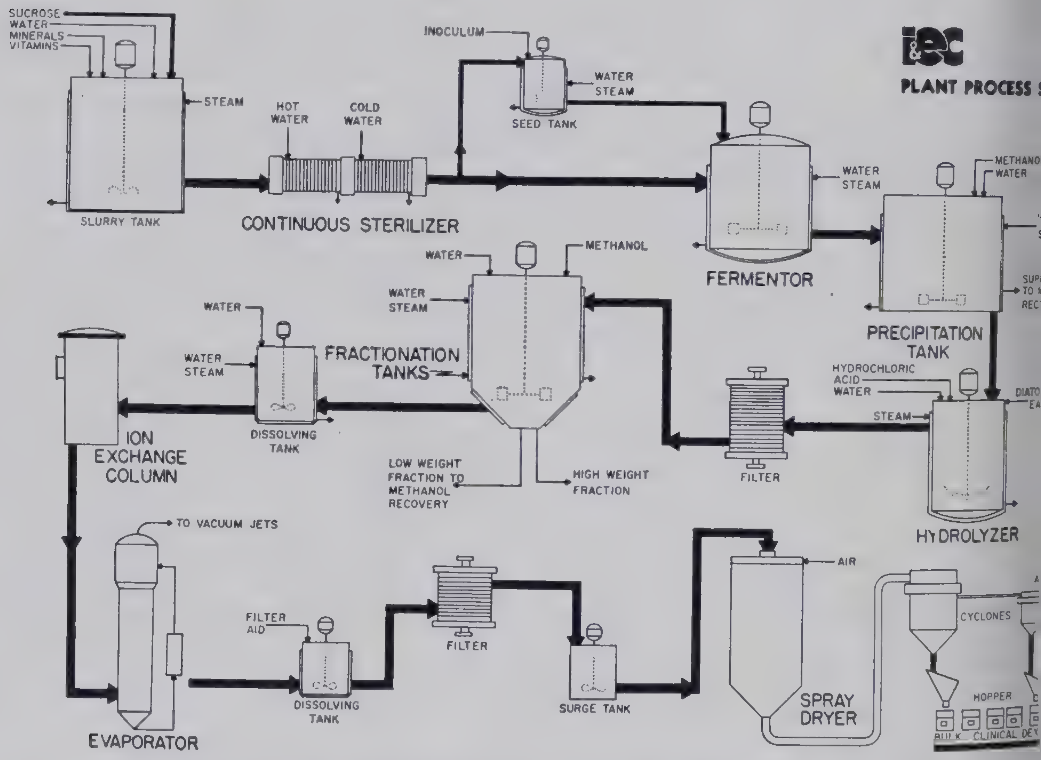
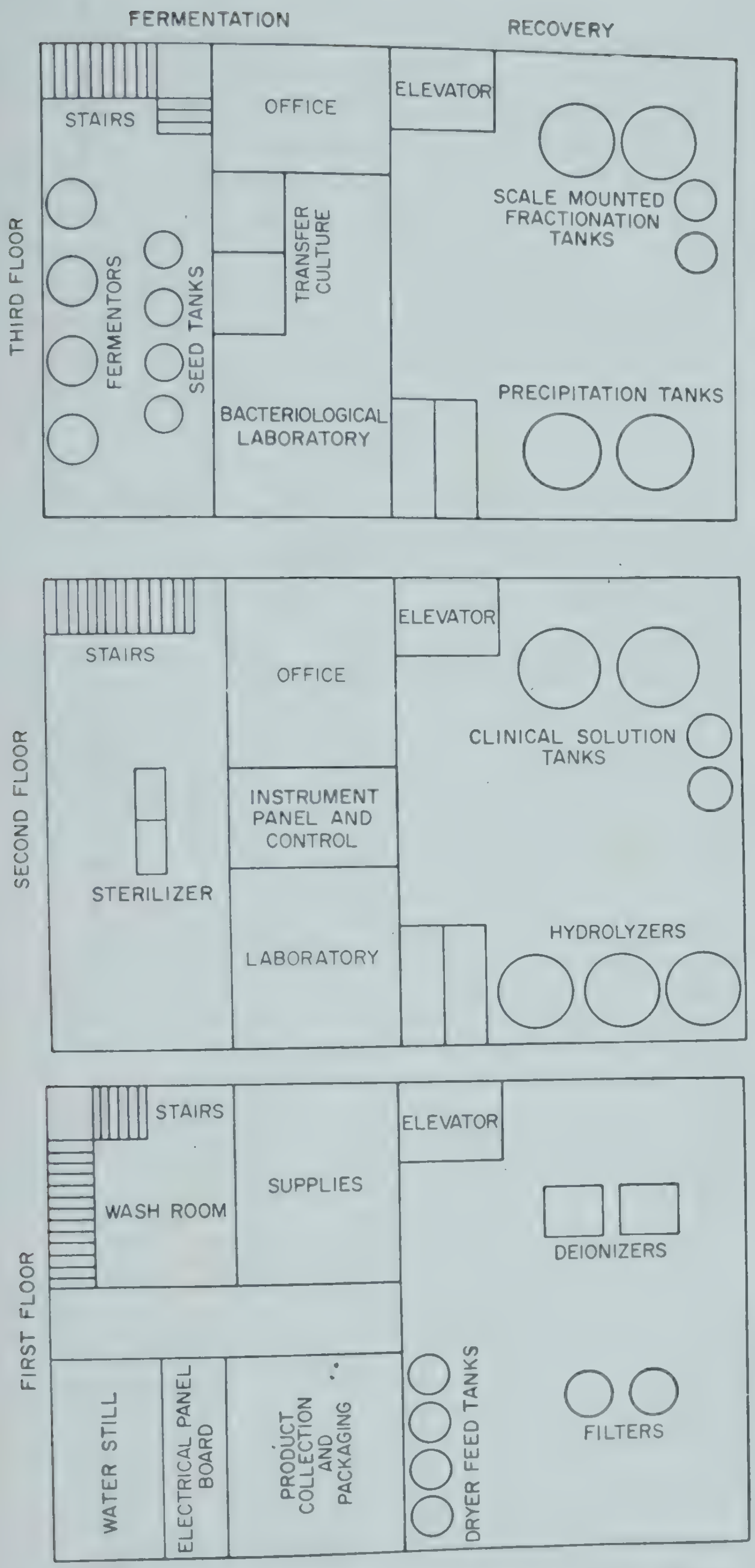

SUCROSE
WATER
MINERALS
VITAMINS
STEAM
SLURRY TANK
HOT WATER
COLD WATER
CONTINUOUS STERILIZER
INOCULUM
WATER
STEAM
SEED TANK
iec
PLANT PROCESS
WATER
STEAM
FERMENTOR
METHANO
WATER
SUP
TO
REC
PRECIPITATION TANK
WATER
METHANOL
WATER
STEAM
WATER
WATER
STEAM
FRACTIONATION TANKS
DISSOLVING TANK
ION EXCHANGE COLUMN
LOW WEIGHT FRACTION TO METHANOL RECOVERY
HIGH WEIGHT FRACTION
FILTER
HYDROCHLORIC ACID
WATER
STEAM
DIATO
EA
HYDROLYZER
TO VACUUM JETS
EVAPORATOR
FILTER AID
DISSOLVING TANK
FILTER
SURGE TANK
AIR
SPRAY DRYER
CYCLONES
HOPPER
BULK
CLINICAL DEX

Fig. 63. Flow sheet for the production of clinical dextran at the Terre Haute. plant of the Commercial Solvents Corp. [*Courtesy of G. H. Bixler, G. E. I R. M. McGhee, and R. A. Shurter, Ind. Eng. Chem.,* 45: 692 (1953). *Copyright by the American Chemical Society and reprinted by permission of the cop owner.*]

unnecessary contamination of the air in the processing building, raw n rials and the slurry tank are located in an adjacent building.

The raw materials are weighed into a 1,500-gal. slurry tank, prod a volume of 1,060 gal. The contents are agitated and their temper


FERMENTATION
RECOVERY
THIRD FLOOR
STAIRS
OFFICE
ELEVATOR
SCALE MOUNTED FRACTIONATION TANKS
FERMENTORS
SEED TANKS
TRANSFER CULTURE
BACTERIOLOGICAL LABORATORY
PRECIPITATION TANKS
SECOND FLOOR
STAIRS
OFFICE
ELEVATOR
CLINICAL SOLUTION TANKS
INSTRUMENT PANEL AND CONTROL
STERILIZER
LABORATORY
HYDROLYZERS
FIRST FLOOR
STAIRS
ELEVATOR
WASH ROOM
SUPPLIES
DEIONIZERS
WATER STILL
ELECTRICAL PANEL BOARD
PRODUCT COLLECTION AND PACKAGING
DRYER FEED TANKS
FILTERS

4. Floor plan of dextran building. [*Courtesy of G. H. Bixler, G. E. Hines, McGhee, and R. A. Shurter, Ind. Eng. Chem.*, **45**: 692 (1953). *Copyright* 1953 *American Chemical Society and reprinted by permission of the copyright* .]

brought to 60°C. through heat supplied in the steam jacket of The solution is pumped to the second floor of the dextran buildi it is sterilized in a two-section, plate-type heat exchanger. The is heated to 142°C. by circulation of water at 150°C. (under pre one side of the plate. In the second section of the heat excha medium is cooled to 25°C. by the use of water at 15°C.

The 1,060 gal. of sterilized medium is pumped aseptically t sterilized system to the third floor of the plant, where it is di between a 1,300-gal. fermentor and a 206-gal. seed tank (both st

The culture is built up in several stages, starting with a pure c the bacteria and using a temperature of 25° ± 0.5°C. A 10-gal. s steel bazooka (refer to Fig. 65) is used for the final stage of i

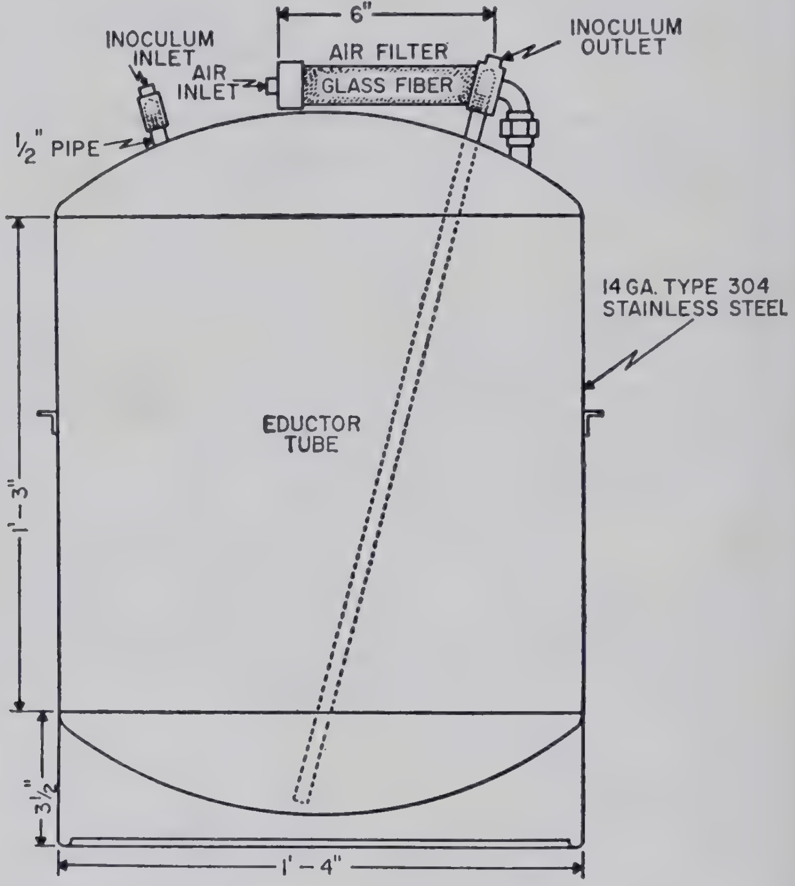


Fig. 65. Bazooka. [*Courtesy of G. H. Bixler, G. E. Hines, R. M. McGh* *R. A. Shurter, Ind. Eng. Chem.*, **45**: 692 (1953). *Copyright* 1953 *by the A* *Chemical Society and reprinted by permission of the copyright owner.*]

built up in the culture room. The temperature of the contents bazooka is maintained at a uniform level by the use of a co temperature bath. The inoculum is transferred from the bazooka seed tank through steam-sterilized, flexible, stainless-steel lines use of sterile air under pressure. Inoculum from the seed tank is quently blown to a 300-gal. fermentor. Each of the latter cont turbine agitator which aids in providing uniform temperature th out the vessel.

uring the fermentation, the pH decreases owing to the production of
nic acids and the viscosity of the medium increases owing to the syn-
is of dextran. In order to assure accurate control, samples are taken
the mash every 2 hr. When the pH has dropped to about 4.5, the
hesis is considered to be complete. At this time, the viscosity may
between 400 and 700 c.p.

ıe spent medium, which contains dextran; unfermented sugars; fer-
ation products, such as acetic and lactic acids, ethanol, and other
tances, is pumped into a 2,400-gal. precipitation vessel, where, after
ng and pH adjustment, an approximately equal amount of methanol
lded (at this stage, the temperature is controlled within a range of
). Nearly all of the dextran is precipitated by the methanol. After
solids have been permitted to settle out, the supernatant liquor is
ped to a 4,000-gal. run-down tank for storage and subsequently to a
ıanol-recovery area.

ıe precipitated native dextran is redissolved in pyrogen-free water
ivalent to double-distilled water, and possessing less than 1.5 p.p.m.
tal solids) at 60 to 70°C. Precipitation of the dextran with methanol
peated to remove impurities occluded in the first precipitate. Super-
nt liquors are pumped also to the run-down tank, and the dextran is
olved again in pyrogen-free water at 60 to 70°C.

ıe dextran solution is pumped to a 1,000-gal. glass-lined hydrolyzer.
ogen-free water is added to adjust the content of dextran. Heat and
um are applied to remove traces of methanol. Hydrochloric acid is
d in measured amount, and the temperature of hydrolysis is kept at
to 105°C. by the application of steam to the jacket of the hydro-
.

e temperature and time of hydrolysis are controlled, based on data
ned by viscosity measurements. Six or more readings are made until
iscosity is approximately 10 c.p. Readings are then taken once every
n., and the data are plotted on semilogarithmic graph paper. The
se end product is predicted from these data. The rate of hydrolysis
owed down by cooling the solution. This is done in such a manner
the viscosity of the solution after cooling and neutralization with
m hydroxide will be less than 5 c.p.

out 35 lb. of diatomaceous earth is added to the hydrolyzed dextran
e hydrolyzer. The material is then pumped through a horizontal
filter press for polishing and hence to an 1,800-gal. fractionation
, which is mounted on a scale capable of measuring net of up to
)0 lb. with an accuracy of 1 lb. The temperature of the tank contents
ntrolled within 1°C. by means of a temperature-control jacket and
ator.

calculated weight of methanol is added to a weighed amount of the
ed dextran solution. Agitation is continued during and after the
ion of the methanol. Dextran, chiefly of high molecular weight, is
pitated. Agitation is stopped, and the precipitate is permitted to
and is then removed. Agitation is started, and an additional charge
ethanol is weighed into the tank. Fractions with molecular weights in

the range of about 25,000 to 200,000 (the clinical range) are precipi this time. They are dropped into a redissolving tank located on the below. The fractions which remain in the supernatant methanol solu largely of low molecular weight, are pumped to the run-down tank methanol recovery.

Pyrogen-free water is added to the clinical-range dextran in the r solving tank to reduce its viscosity for pumping. This material is pun to a 750-gal. fractionation tank, which is mounted on weighing scales is located on the third floor of the plant. Here fractionation into high- clinical-range fractions is repeated. The clinical material is dropped 300-gal. redissolving tank on the second floor. Pyrogen-free wate added to reduce the viscosity to the point where the solution will readily through a mixed-fed deionizing column. The deionized dex solution is pumped to a tube evaporator possessing 60 sq. ft. of surfac heat transfer. Filter aid (about 0.5 per cent) is added to and mixed the concentrated solution in a dissolving tank. The mixture is polis by passage through a filter. The clear solution is pumped to a 100- spray-drier feed tank.

Spray drying represents the final stage of processing. The concentra dextran solution is fed through an atomizing disk spinner, 5 in. in dia eter, which is located at the top of a 7-ft. drying chamber. The rotates at a rate of 21,000 r.p.m. and disperses the dextran solution hot gases. The dextran remains in contact with the heating medium about 5 sec., and its temperature does not exceed 92.2°C. The solut is reduced to a powder (with a moisture content of about 1 per cent) a collected in a series of four cyclone separators, which are mounted parallel. Material from the cyclones is dropped to a central hopper packing.

Dextran is highly hygroscopic. Consequently it must be packed in co tainers which provide adequate protection against moisture absorpti Fiber drums lined with polyethylene, containing a barrier of alumin in the wall, and equipped with snap lids with rubber gaskets have be used.

The bulk material is held until laboratory tests indicate that it me specifications in all respects. Thereafter it may be released for bottli an operation which may be carried out at a separate plant.

The dried dextran is dissolved in pyrogen-free water, its concentrati is adjusted to 6 per cent (6 g. per 100 ml.), sodium chloride is added provide a concentration of 0.9 per cent, and the solution is filtered. Suf cient of this solution is introduced into a plasma-type bottle to ensu delivery of a 500-ml. amount. The air is withdrawn from the bottle, whic is sealed and capped. The bottle with its contents is heat-sterilized. It packaged with the required equipment for injection.

**Specifications.** The bottled dextran must meet the Armed Forc specifications, which are given below, as well as additional specificatio pertaining to color and methanol content. The color must be less tha APHA 50, and the methanol content must not exceed 20 mg. per 100 ml.

Table 107. Specification for Dextran *

| Property | Value for 6% Solution |
|---|---|
| an content, g./100 ml. | 5.7 –6.3 |
| m chloride, g./100 ml. | 0.85–0.95 |
|  | 5.0 –7.0 |
| sity, c.p. at 25°C. | 2.5 –3.5 |
| gen (max.), mg./100 ml. | 1.0 |
| y metals (max. as Pb), mg./100 ml. | 0.5 |
| max. residue on ignition), mg./100 ml. | 0.056 |
| nt average: † |  |
| lecular weight | 75,000 ± 25,000 |
| he high fraction (5–10%) shall not exceed 200,000; |  |
| low fraction (5–10%) shall not be less than 25,000.. |  |
| sic viscosity deciliter/g. | 0.23 ± 0.05 |
|  | Colorless–light |
| ing capacity (max.), ml. of 0.100 *N* NaOH/100 ml. |  |
| dextran solution | 3.0 |
| um in bottle (min.), in. of Hg. | 15 |
| ity | Negative |
| genicity | Negative |
| enicity | Negative |
| ity | Negative |

rmed Forces Medical Procurement Agency, Specification 1-161-890, May 21.

etermined by light-scattering measurements.

## PRODUCTION OF DEXTRAN BY ENZYMATIC SYNTHESIS

e fundamental studies of Hehre (1941, 1946, 1951) and of Hehre and
(1942) on dextransucrase laid the foundation for the extensive
rch on the enzymatic synthesis of dextran carried out by the scien-
of the Northern Utilization Research and Development Division of
.S. Department of Agriculture under the direction of Jackson and
s and by other agencies.

**ocess in Brief.** The process for the enzymatic synthesis of dextran
ves (1) the production of dextransucrase; (2) the removal of the
rial cells from the production medium; (3) the synthesis of dextran
reaction mixture containing sucrose, dextransucrase, and a primer
carefully controlled conditions; and (4) fractionation and purifica-
of the dextran (refer to Fig. 66 for flow sheet).

**xtransucrase Production.** Koepsell and Tsuchiya (1952) reported
dextransucrase could be formed rapidly and in high yield.

uchiya et al. (1952) described the effects of various cultural condi-
on dextransucrase production by *Leuconostoc mesenteroides* NRRL
2. They developed a medium for the production of the enzyme which
ined 2 per cent sucrose, 2 per cent corn-steep liquor (dry basis),
r cent potassium dihydrogen phosphate, and 0.5 per cent by volume
ts (4 per cent magnesium sulfate heptahydrate, 0.2 per cent sodium
ide, 0.2 per cent ferrous sulfate heptahydrate, and 0.2 per cent man-
us sulfate monohydrate).

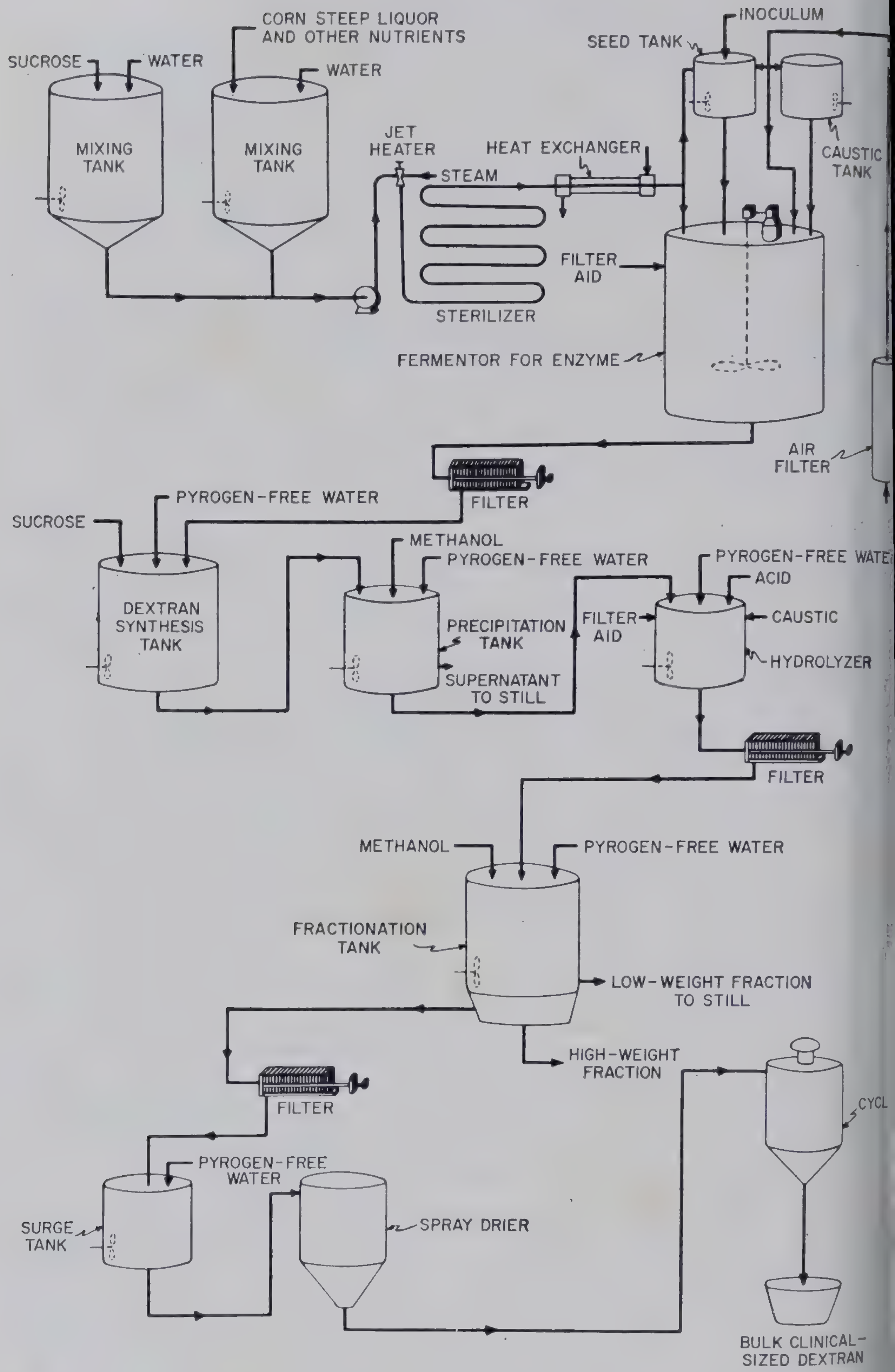
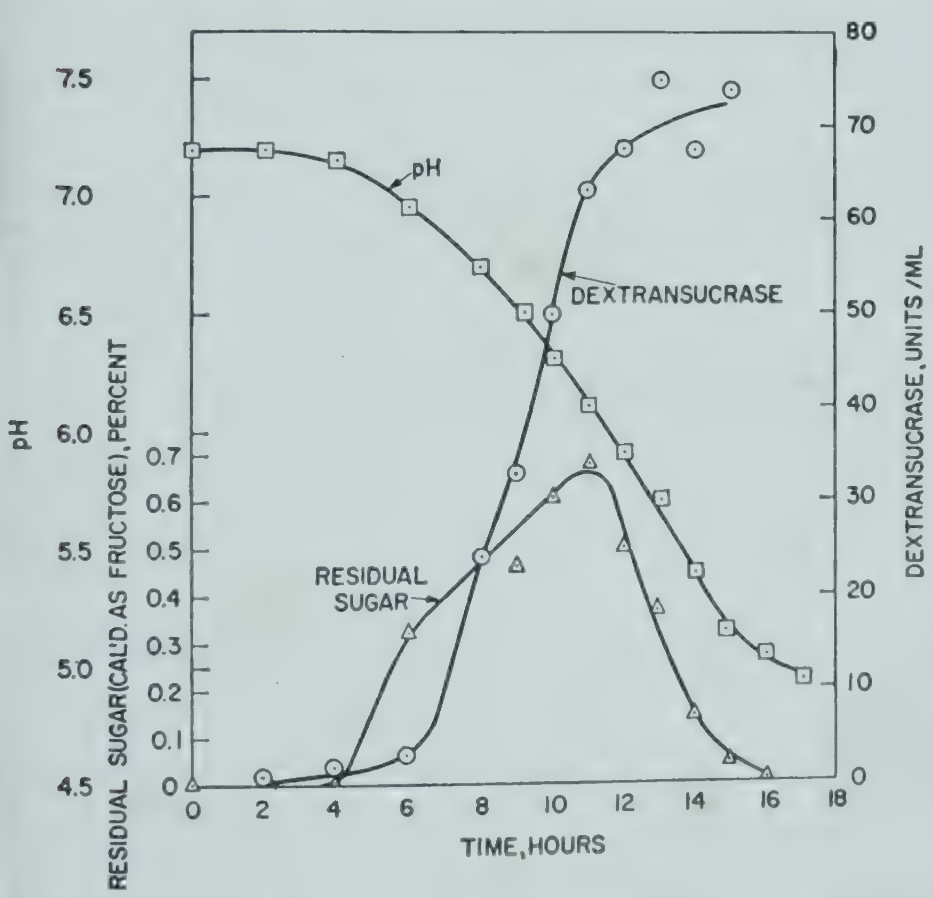

SUCROSE
WATER
CORN STEEP LIQUOR AND OTHER NUTRIENTS
WATER
MIXING TANK
MIXING TANK
JET HEATER
STEAM
HEAT EXCHANGER
INOCULUM
SEED TANK
CAUSTIC TANK
STERILIZER
FILTER AID
FERMENTOR FOR ENZYME
AIR FILTER
FILTER
PYROGEN-FREE WATER
SUCROSE
DEXTRAN SYNTHESIS TANK
METHANOL
PYROGEN-FREE WATER
PRECIPITATION TANK
SUPERNATANT TO STILL
FILTER AID
PYROGEN-FREE WATE
ACID
CAUSTIC
HYDROLYZER
FILTER
METHANOL
PYROGEN-FREE WATER
FRACTIONATION TANK
LOW-WEIGHT FRACTION TO STILL
HIGH-WEIGHT FRACTION
CYCL
FILTER
PYROGEN-FREE WATER
SURGE TANK
SPRAY DRIER
BULK CLINICAL-SIZED DEXTRAN

FIG. 66. Flow sheet for the production of clinical-sized dextran by enzymatic sy thesis and acid hydrolysis. [*Courtesy of V. E. Sohns, S. P. Rogovin, H. F. Con and C. T. Langford, U.S. Dept. Agr., Bur. Agr. Ind. Chem., Mimeographed Circ. S AIC 372, June,* 1954.]

hey established optimum conditions for the production of the dex-
sucrase in this medium. The pH was best when maintained at 6.7 by
ıddition of sodium hydroxide or potassium hydroxide. Unfortunately
was not the optimum pH for the retention of the enzymatic activity,
as much as 35 per cent of the activity may be lost in 1 hr. at 25°C.
ever, at a pH of 5 to 5.2 the enzymatic activity is most stable (Koep-
and Tsuchiya, 1952) and may be retained in culture filtrates for
r. or longer at the same temperature. A temperature of 25 ± 1°C.
used for the production of the enzyme. Higher temperatures accel-
d the destruction of the activity. Mild aeration favored the produc-
of the dextransucrase. This could be obtained on shakers or by
ing 0.05 volume of air per volume of medium per minute through the
trate with moderate agitation. Ammonium ions exerted an adverse
t on enzyme production; accordingly, ammonium hydroxide should
e used to neutralize the medium. A sucrose concentration of 2 per
appeared to be optimum, for higher concentrations increased the
unt of dextran and the difficulty of removing the bacterial cells. Cell-
liquors were obtained by passing the centrifuged material through a
filter. However, centrifuging alone proved to be satisfactory when
noved substantially all the cells.

gure 67 illustrates the production of dextransucrase as a function



7. The formation of dextransucrase. [*Courtesy of H. M. Tsuchiya, H. J.
ell, J. Corman, G. Bryant, M. O. Bogard, V. H. Feger, and R. W. Jackson,
Bact.,* **64**: 521 (1952).]

of pH, residual sugar, and time from a sucrose–corn-steep-liquo
medium.

Sohns et al. (1954) described the pilot-plant production of cli
sized dextran by acid hydrolysis of the enzymatically synthesize
polymer. The discussion which follows is based largely on their repo

The culture of *L. mesenteroides* NRRL B-512F was carried on a
medium, the composition of which is given below. Cultures were
for 18 to 24 hr. at 25°C. and stored until used at about 4°C. New cu
were made up once a month.

The development of the inoculum was initiated by transferring
from the slant culture to a 300-ml. Erlenmeyer flask which cont
100 ml. of the inoculum development medium shown below and by
bating this flask on a reciprocating shaker for approximately 16 hr.

| | Slant medium * | Inoculum develop medium * |
|---|---|---|
| Sucrose | 2.0% | 2.0% |
| Yeast extract (Difco) | 0.5% | 0.5% |
| Dibasic potassium phosphate | 0.25% | 0.5% |
| Tryptone (Difco) | 0.25% | 0.25% |
| Agar | 2.0% | None |
| Initial pH (approximately) | 7.4 | 7.4 |

* V. E. Sohns, S. P. Rogovin, H. F. Conway, and C. T. Langford, *U.S. Dept.*
*Bur. Agr. and Ind. Chem., Mimeographed Circ. Ser., AIC* 372, 1954.

A medium known as the "Basamin-Busch medium," the compositio
which follows, was developed by the Northern Utilization Resea
and Development Division of the U.S. Department of Agriculture
Peoria, Ill. (Jackson, 1956):

| | |
|---|---|
| Sucrose | 3.0% |
| Basamin-Busch yeast hydrolyzate | 0.5% |
| $K_2HPO_4$ | 3.0% |
| $MgSO_4 \cdot 7H_2O$ | 0.02% |
| NaCl | 0.001% |
| $FeSO_4 \cdot 7H_2O$ | 0.001% |
| $MnSO_4 \cdot H_2O$ | 0.001% |
| Initial pH | 7.4 |

This medium may be used for laboratory-scale fermentations or
developing inocula for pilot-plant applications.

The Basamin-Busch medium may be modified to serve as a substr
for pilot-plant fermentations by making the following changes (Jacks
1956): The concentration of $K_2HPO_4$ is reduced from 3 to 0.1 per ce
The pH, which is initially 7.4, drops to 7 soon after inoculation and
maintained at this level by the automatic addition of sodium hydrox
until the fifth or sixth hour, after which there is no further addition
the alkali. The pH decreases in a gradual manner to 5.7 to 5 at the te
to eleventh hour, when the fermentation is considered to be complete.

building up the inoculum or starter, the usual practice was to seed larger volume with 5 per cent of inoculum. The final fermentation um received 1 to 2 per cent of inoculum.

e temperature used in producing dextransucrase was 25°C.

ld aeration was supplied by agitating the flasks or by sparging air he medium. In the pilot-plant fermentor, sterile air at a pressure of 4 lb. per sq. in. (gauge) was maintained above the surface of the ım, which was agitated during the fermentation. All of the air used terilized.

e cultures were usually grown for about 12 hr. during each stage in uild-up of the starter. The final fermentation is of shorter duration nay be terminated after 10 to 11 hr., or less.

ıns et al. (1954) sterilized some of the media continuously for 3 min. emperature of 135°C.

the time the fermentation is completed, there should be about xtransucrase units per ml. of liquor. A unit of dextransucrase is the nt of the enzyme which will convert 1 mg. of sucrose to dextran in at 30°C. and at pH 5 in a reaction mixture containing 10 per cent se, $M/20$ acetate buffer, and the centrifuged culture (Koepsell and iiya, 1952).

**noval of Bacteria.** If absolute removal of the cells is desired, it be accomplished by the use of a Seitz or membrane or molecular In practice, removal of cells is carried out by centrifuging. Cells ıspended matter may also be removed by filtration methods.

er removal of the cells and suspended matter, the pH of the filtrate usted immediately to 5 to 5.2, the optimum for stability and activ- The enzyme-containing liquor may be stored at this pH and at a rature of about 15°C. for 30 days or longer without appreciable loss vity, according to Sohns et al. (1954).

**thesis of Dextran.** The following factors are among those con- in the controlled synthesis of dextran: (1) the concentration of e, (2) the concentration of dextransucrase, (3) the type of primer, e concentration of the primer, and (5) the reaction temperature. istribution of molecular weights is influenced but slightly within I range of 4.7 to 5.6 (Tsuchiya et al., 1955).

hiya and associates (1955) reported that the initial sucrose con- tion of the reaction mixture influenced the yield and the molecular of the synthesized dextran. When sucrose concentrations were sed from 5 to 20 per cent, the yield of dextran was decreased as well molecular weight. The dextran formed at a sucrose concentration per cent was of low molecular weight, whereas the main product at a sucrose concentration of 10 per cent was of high molecular . When the concentration of sucrose is increased, the concentration tose also increases, as does the total amount of primer.

enzyme concentrations ordinarily used contain 20 to 40 dextran- units per ml. Increasing the enzyme concentration at about 30°C. the production of dextran of low molecular weight.

psell et al. (1952, 1953) indicated that certain sugars and sugar

derivatives may function in the role of glucosyl acceptors or pr
Maltose, isomaltose, alpha-methyl glucoside, dextran of low mol
weight, and other substances may serve as primers with different r

Maltose induced the formation of oligosaccharides and dextran
molecular weight (Tsuchiya et al., 1955). The distribution of mol
weights was wide, and the yield of clinical-sized product was low.

The reaction of maltose as a glucosyl acceptor has led to the de
ment of a method for producing panose, a 4-α-isomaltopyranosyl-
cose, through the action of NRRL B-512F dextransucrase on a m
of sucrose and maltose. The method for preparation has been des
by Killey, Dimler, and Cluskey (1954).

Dextran of low molecular weight is superior to maltose for the pr
tion of dextran of a molecular weight in the clinical range (75,0
25,000). Dextran for primer use may be obtained as a by-product
recovery process for clinical dextran. The fraction with a mole
weight below the clinical range or the hydrolyzed dextran of a mole
weight above this range may be used for primer purposes. In genera
increase in the molecular weight of the primer increases the mole
weight of the synthesized dextran. According to Hellman and assoc
(1955), suitable weight-average primer molecular weights for the pro
tion of dextran of molecular weight 75,000 ± 25,000 are in the rang
15,000 to 20,000 for dextran fractions and 20,000 to 40,000 for dex
hydrolyzates.

Dextran of 75,000 ± 25,000 molecular weight has been obtaine
yields of more than 25 per cent of the reacted sucrose, when a 2 per
concentration of dextran primer with a molecular weight of near 20
was used and when the reaction mixture was fractionated between
limits of 42 and 50 per cent actual concentration of methanol (Hell
et al., 1955).

The temperature of the enzyme reaction affects the molecular-we

TABLE 108. INFLUENCE OF REACTION TEMPERATURE ON DEXTRAN-PRIMED ENZYMATIC SYNTHESIS *, †

| Temperature, °C. | Yield, % of reaction sucrose | | |
|---|---|---|---|
| | Total ‡ dextran | Low-mol.-wt. range ‡ | High-mol.-wt. range |
| 30 | 45.9 | 21.2 | 24.7 |
| 15 | 47.4 | 43.4 | 4.0 |
| 4 | 47.2 | 45.9 | 1.3 |

* N. N. Hellman et al., *Ind. Eng. Chem.*, 47: 1593 (1955). Copyright 1955 by
American Chemical Society and reprinted by permission of the copyright ow

† Reaction conditions: sucrose concentration, 10 per cent; primer-dextran fra
of molecular weight, 21,000 at 1 per cent concentration; enzyme concentration
dextran-sucrase units per ml.

‡ Yields determined from plateau or inflection in function of dextran precipit
versus amount of methanol added to reaction mixture.

bution of the dextran, the reaction rate, and the stability of the
ne. Tsuchiya et al. (1955) found that the production of low modal
molecular-weight) dextran was enhanced by increasing the enzyme
ntration at 30°C. or by decreasing the temperature of reaction. The
nce of the reaction temperature on the yield of low- and high-
ular-weight dextran is shown in Table 108.

us high yields of products with molecular weights satisfactory for
al use have been obtained by carrying out the reaction at about
, or less, and by employing a sucrose concentration of 10 per cent,
zyme concentration of 20 to 40 dextransucrase units per ml., and
olecular-weight dextran as a primer.

er the synthesis is complete, methanol is added until its concentra-
s 50 per cent by volume, for the purpose of precipitating the dextran.
nixture is agitated during the addition to prevent local high con-
tions of the alcohol. About 5 hr. is allowed for the precipitated
n to settle. The supernatant liquor, which contains the fractions of
olecular weight, is diverted to a methanol-recovery area. The crude
n is dissolved in pyrogen-free water, and methanol is added to
a concentration of 50 per cent by volume. The dextran is reprecipi-
This material requires no further reprecipitation before hydrolysis
ractionation, according to Sohns and associates (1954). Either the
dextran or the reprecipitated product may be processed to yield
l fractions meeting the specifications of the Armed Forces Medical
rement Agency.

**cessing of Clinical Dextran.** The pilot-plant processing of dextran
d by one reprecipitation, as reported by Sohns et al. (1954) and
strated by the flow sheet shown in Fig. 68, is briefly described below.

reprecipitated dextran (29.2 lb.) is dissolved in pyrogen-free water
d a dextran concentration of 5 to 6 per cent. The pH of the solu-
adjusted to 1.05 with 6$N$ $H_2SO_4$. The solution is heated to 80°C.
aintained at this temperature with mild agitation during the
ysis of the dextran. Water, lost by evaporation, is replaced as
d. The progress of the hydrolysis is determined by measurements
relative viscosity. After the latter has dropped to the desired
2.66 measured at 25°C., in this example), which requires about
he pH is raised at once to 7.0 with caustic and the temperature is
d to that of the room by rapid cooling. Water is added to yield a
cent concentration of dextran. The solution is filtered. Methanol
ed slowly to the vigorously agitated solution until its concentration
s 42 per cent by volume at 25°C. Addition of the alcohol causes a
the temperature of the reaction mixture, but it is adjusted to 25°C.
ach addition. A dextran of high molecular weight is precipitated,
ting to about 1.5 lb. (dry basis). The supernatant, which contains
27.2 lb. of the clinical fraction on a dry basis, is decanted and
l through a horizontal plate filter. The concentration of alcohol in
rate is brought to 49 per cent, which results in the precipitation
lb. of crude clinical fraction.

supernatant is decanted, and the fraction is dissolved in water to

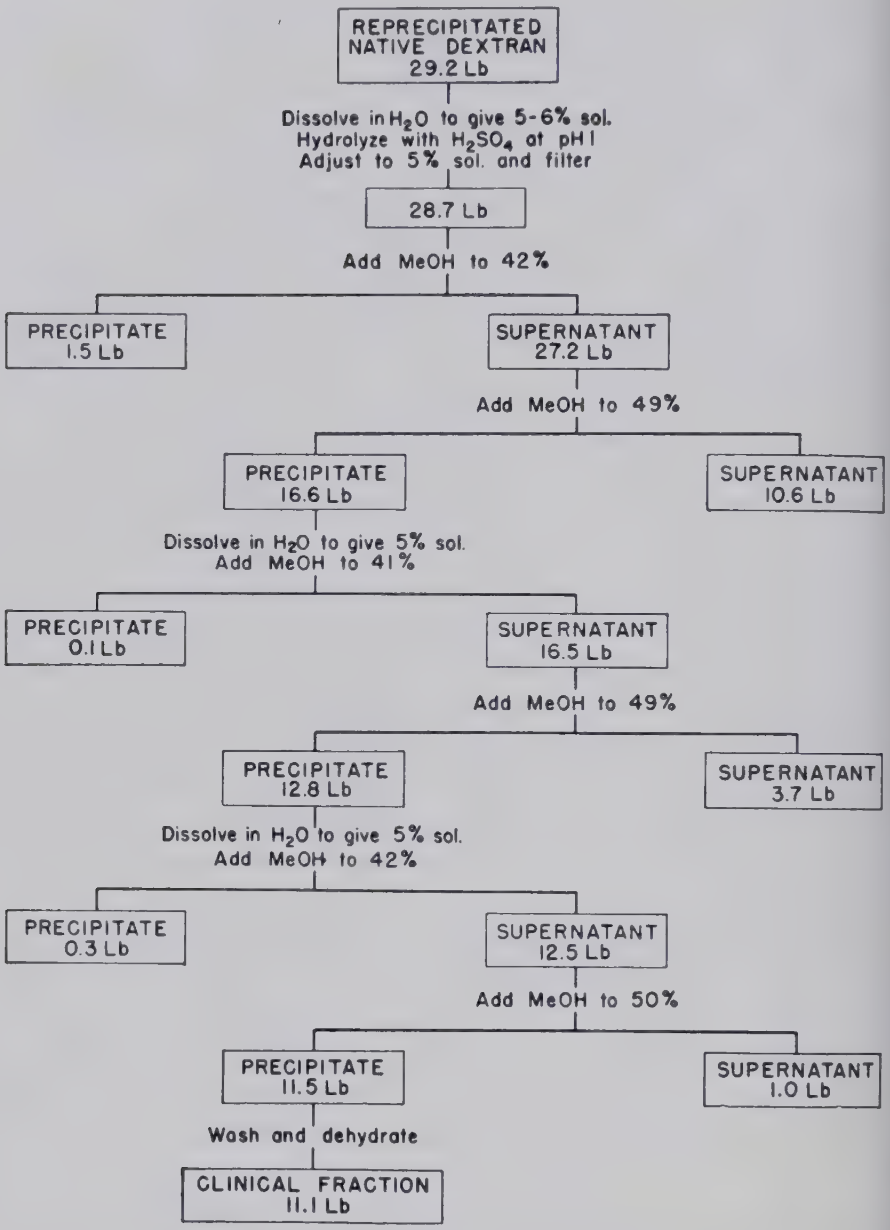

REPRECIPITATED NATIVE DEXTRAN 29.2 Lb
Dissolve in $H_2O$ to give 5-6% sol.
Hydrolyze with $H_2SO_4$ at pH 1
Adjust to 5% sol. and filter
28.7 Lb
Add MeOH to 42%
PRECIPITATE 1.5 Lb
SUPERNATANT 27.2 Lb
Add MeOH to 49%
PRECIPITATE 16.6 Lb
SUPERNATANT 10.6 Lb
Dissolve in $H_2O$ to give 5% sol.
Add MeOH to 41%
PRECIPITATE 0.1 Lb
SUPERNATANT 16.5 Lb
Add MeOH to 49%
PRECIPITATE 12.8 Lb
SUPERNATANT 3.7 Lb
Dissolve in $H_2O$ to give 5% sol.
Add MeOH to 42%
PRECIPITATE 0.3 Lb
SUPERNATANT 12.5 Lb
Add MeOH to 50%
PRECIPITATE 11.5 Lb
SUPERNATANT 1.0 Lb
Wash and dehydrate
CLINICAL FRACTION 11.1 Lb

Fig. 68. Quantitative flow sheet for producing clinical-sized dextran from the cipitated native product. [*Courtesy of V. E. Sohns, S. P. Rogovin, H. F. Co and C. T. Langford, U.S. Dept. Agr., Bur. Agr. Ind. Chem., Mimeographed Circ AIC* 372, *June,* 1954.

yield a 5 per cent solution. Methanol is added to produce a volum 41 per cent, which results in the precipitation of 0.1 lb. of material. supernatant is decanted and filtered, and its alcohol content is broug 49 per cent by volume. A precipitate weighing 12.8 lb. results.

The supernatant is decanted, and the precipitate is dissolved in to produce a 5 per cent solution. Fractionations are made at 42 an per cent methanol concentrations, successively. A yield of 11.1 l clinical dextran is obtained after the 11.5 lb. of material precipitate the 50 per cent methanol is washed and dried. This amount of mat represents a yield of 38 per cent, based on the native dextran, or 12. cent, based on the sugar used.

## HODS FOR THE PARTIAL DEPOLYMERIZATION OF DEXTRAN

xtran may be depolymerized by acid, alkaline, or enzymatic hydroly- y heat; by ultrasonic vibrations; and by ultraviolet light.

id hydrolysis appears to be the most attractive method because it res no complex equipment and may be controlled readily. Colin and l (1940) described the partial hydrolysis of dextran with acid and recipitation of the hydrolyzed product with alcohol. Grönwall and man (1948, 1953) and Lockwood and Swift (1951) reported on the of lower-molecular-weight alcohols, acetone, and dioxane for the onation of partial dextran hydrolyzates. Two particularly valuable ts were made by Wolff and associates in 1954 concerning the produc- of clinical-type dextran. One of these reports dealt with the partial lytic depolymerization and fractionation of the dextran from *Leu- toc mesenteroides* NRRL B-512; the other dealt with the dextrans *L. mesenteroides* NRRL B-742 and *Streptobacterium dextranicum* L B-1254. Among the effects studied were the effects of pH-tem- ure-time relationships during depolymerization, the kind of acid, t of hydrolysis, dextran concentration, and variation in conditions of onation on the course of the degradation of dextran, on the yield, n the properties of the clinical-type fraction. The authors found "careful control of the extent of hydrolysis by interrupting the ion when a predetermined viscosity" had been reached was "more tant than the choice of the acid or of any particular set of hydroly- nditions."

**polymerization by Use of Enzymes from Mold.** Charles and Far- 1957) reported on the preparation and use of enzymatic material *Penicillium lilacinum* to obtain clinical dextran by hydrolysis of the ntation broth of *L. mesenteroides* NRRL B-512. The best medium production of the extracellular enzyme contained 2 per cent native n and 2 per cent corn-steep solids. This was sterilized in 100-ml. ts in 500-ml. Erlenmeyer flasks for 25 min. at 121°C. After cooling, ntents were inoculated with spores (about $10^8$), which had been on potato dextrose agar for 8 days at 23°C., and incubated at 23°C. otary shake table operated at 300 r.p.m. for 8 days.

enzyme was most active, and apparently independent of pH, in the nge of 4.75 to 6.5. Activity declined gradually at a pH above 6.5 ropped sharply at a pH below 4.5. Heating of the crude enzyme e at 60°C. for 30 min. at pH levels of 6 to 8.5 showed that the e system was stable in this range; however, at pH 5 the activity lmost completely arrested.

optimum yield of clinical dextran obtained by enzymatic cleavage nted to 55 to 60 per cent of the native dextran present in the fermen- broth. The procedure followed for obtaining the clinical dextran sentially as follows: The contents of the fermentation tank were l to 80°C. at the end of the fermentation and then cooled to 23°C. H was adjusted to 6, and 80 units of enzyme were added for each liter of fermentation medium. The mixture was agitated until the re viscosity reached 2.4. Acid was added to the reaction mixture to pr a pH of 2.5, and the temperature was raised to 40°C. to inactivat enzyme. The pH was then adjusted to 6, and the mixture was frac ated with alcohol.

The clinical dextran produced by this method complied with the and Drug Administration directorate and was used clinically.

Some references relating to some methods which may be use degrading dextrans are presented in Table 109.

Table 109. Some Data concerning Some Methods Which May Be Use for Degrading Dextrans

| Method | References |
|---|---|
| Alkaline hydrolysis | Stacey, M.: Abstr. of Papers, 12th *I* *Congr. Pure Appl. Chem.,* New Sept. 10–13, 1951, p. 623. |
| Enzymatic hydrolysis | Ingelman, B.: *Acta Chem. Scand.,* **2** (1948).<br>Nordstrom and Hultin: *Svensk Kem.* **60**: 283 (1948).<br>Hultin and Nordstrom: *Acta Chem. Sc* **3**: 1405 (1949).<br>Tsuchiya, H. M., J. Corman, and M gomery: *Jour. Am. Chem. Soc.,* **71**: (1949).<br>Hehre, E. J., and Sery: *Jour. Bact.,* **63**: (1952).<br>Whiteside-Carlson, V., and W. W. Carl *Science,* **115**: 43 (1952).<br>Tsuchiya, H. M., A. Jeanes, H. M. Bri and C. A. Witham: *Jour. Bact.,* **64**: (1952).<br>Charles, A. F., and L. N. Farrell: *Can. J* *Microbiol.,* **3**(2): 239 (1957). |
| Thermal depolymerization | Stacey, M., and Pautard: *Chemistry* *Industry,* 1952, p. 1058.<br>Wolff, I. A., P. R. Watson, J. W. Sloan, C. E. Rist: *Ind. Eng. Chem.,* **45**: (1953). |
| Ultrasonic-vibrations degradation | Lockwood, Jones, and Pautard: *Rese* *(London),* **4**: 46 (1951).<br>Stacey, M.: *Research (London),* **4**: (1951).<br>Pautard: *Chemistry & Industry,* 1953, 1316.<br>Watson, P. R., and I. A. Wolff: *Jour.* *Chem. Soc.,* **77**: 196 (1955). |
| Ultraviolet-light degradation | Watson, P. R., and I. A. Wolff: *Jour.* *Chem. Soc.,* **77**: 196 (1955). |

## APPLICATIONS OF DEXTRANS

though interest in dextran has centered in its use as a blood volume nder, various other applications have been developed. Patents have issued covering a number of uses, for example, as a method of pre- ng shrimp (Toulmin, Jr., 1956), as a method of preserving food ucts in which the dextran serves as a protective film (Toulmin, ), as a food preservative in which dextran and an antibiotic are ›ined (Novak, 1957), in the manufacture of coating compounds ılmin, Jr., 1956), in the making of special filaments (Deniston, 1955; ɔp, 1957), for the forming of shaped articles (Hiller, 1957), for icing ›ositions (Toulmin, 1957), and in lipstick manufacture (Novak and e, 1956).

### Some References concerning Dextrans

vmous: Blood Plasma Substitute (a Flowsheet), *Chem. Eng.*, December, 1952, p. 240–243.

-Arcega, L., and F. M. Yenko: Some Factors Affecting Production of Dextran om Cane Sugar by *Leuconostoc dextranicum*, *Philippine Jour. Sci.*, **77**: 39–52 1947).

, G. H., G. E. Hines, R. M. McGhee, and R. A. Shurter: Dextran, *Ind. Eng. hem.*, **45**: 692–705 (1953).

es, A. F., and L. N. Farrell: Preparation and Use of Enzymatic Material from . *lilacinum* to Yield Clinical Dextran, *Can. Jour. Microbiol.*, **3**: 239–247 1957).

man, W. L.: Blood and Blood Plasma Substitutes, *U.S. Dept. Agr., Bur. Agr. nd Ind. Chem., Mimeographed Circ. Ser., AIC* 289, October, 1950.

H., and H. Belval: Sur la constitution du dextrane, *Compt. rend. acad. Sci. Paris)*, **210**: 517–520 (1940).

, W. D., and M. Stacey: The Polysaccharide Produced from Sucrose by *Beta- cterium vermiforme*, *Biochem. Jour.*, **32**: 1946 (1938).

r, R. J., H. A. Davis, G. J. Gill, and C. E. Rist: Analytical Total Acid Hydroly- ; of Dextrans, *Anal. Chem.*, **26**: 1142–1146 (1954).

h, W. G. C., and D. M. Webley: The Reducing Sugars Liberated during the ıcterial Synthesis of Polysaccharides from Sucrose, *Jour. Gen. Microbiol.*, 87 (1950).

all, A.: "Dextran and Its Use in Colloidal Infusion Solutions," Academic Press, c., New York, 1957.

and B. G. A. Ingelman: *Acta Physiol. Scand.*, **7**: 97 (1944).

and ———: *Acta Physiol. Scand.*, **9**: 1 (1945).

and ———: U.S. Patent 2,437,518, Mar. 9, 1948.

and ———: U.S. Patent 2,644,815, July 7, 1953.

th, W. N., and M. Stacey: Improvements Relating to the Production of Dex- ın from Sucrose by Means of Dextran-forming Bacteria, British Patent 618,999, ar. 2, 1949.

, E. J.: *Science*, **93**: 237 (1941).

: Studies on the Enzymatic Synthesis of Dextran from Sucrose, *Jour. Biol. hem.*, **163**: 221 (1946).

: An Unusual Streptococcus That Yields Low Molecular Weight (Clinical pe) Dextran Directly, *Bact. Proc.*, 1952, p. 23.

: Natural Synthesis of Low Molecular Weight (Clinical Type) Dextran by a eptococcus Strain, *Jour. Biol. Chem.*, **222**: 739–750 (1956).

and J. Y. Sugg: *Jour. Expt. Med.*, **75**: 339 (1942).

n, N. N., H. M. Tsuchiya, S. P. Rogovin, B. L. Lamberts, R. Tobin, C. A. ıss, C. S. Stringer, R. W. Jackson, and F. R. Senti: Controlled Enzymatic

Synthesis of Dextran, Conditions for Producing Clinically Suitable M Weight, *Ind. Eng. Chem.*, **47**: 1593–1598 (1955).

Hestrin, S., and S. Amineri-Shapiro: The Mechanism of Polysaccharide Pr from Sucrose, *Biochem. Jour.*, **38**: 2–10 (1944).

Hucker, G. J., and C. S. Pederson: The Genus *Leuconostoc, New York St Expt. Sta., Tech. Bull.* 167, 1930.

Ingelman, B.: Enzymatic Breakdown of Dextran, *Acta Chem. Scand.*, **2**: (1948).

Jackson, R. W.: Private Communication, 1956.

Jeanes, A., W. C. Haynes, and C. A. Wilham: Characterization of Dextra Four Types of *Leuconostoc mesenteroides, Jour. Bact.*, **71**: 167–173 (195

———, ———, ———, J. C. Rankin, E. H. Melvin, M. J. Austin, J. E. C B. E. Fisher, H. M. Tsuchiya, and C. E. Rist: Characterization and Classi of Dextrans from Ninety-six Strains of Bacteria, *Jour. Am. Chem. S* 5041–5052 (1954).

———, N. C. Schieltz, and C. A. Wilham: Molecular Association in Dextran Branched Amylaceous Carbohydrates, *Jour. Biol. Chem.*, **176**: 617–627 (

——— and C. A. Wilham: Periodate Oxidation of Dextran, *Jour. Am. Chen* **72**: 2655–2657 (1950).

——— and ———: Dextran Triacetates, *Jour. Am. Chem. Soc.*, **74**: 5339 (195

———, ———, and G. E. Hilbert: Acetobrominolysis of Di- and Polysacchari tates, *Jour. Am. Chem. Soc.*, **75**: 3667–3673 (1953).

———, ———, R. W. Jones, H. M. Tsuchiya, and C. E. Rist: Isomaltase a maltotriose from Enzymic Hydrolyzates of Dextran, *Jour. Am. Chem. S* 5911–5915 (1953).

———, ———, and J. C. Miers: Preparation and Characterization of Dextra *Leuconostoc mesenteroides, Jour. Biol. Chem.*, **176**: 603–615 (1948).

——— with the assistance of L. Huntsinger and N. G. Larson: Dextran—A S Bibliography, *U.S. Dept. Agr., Bur. Agr. and Ind. Chem., Mimeographe Ser., AIC* 288 (revised), June, 1952.

Killey, M., R. J. Dimler, and J. E. Cluskey: Preparation of Panose by the Ac NRRL B-512 Dextransucrase on a Sucrose-Maltose Mixture, *Jour. Am. Soc.*, **77**: 3315 (1955).

Koepsell, H. J., and H. M. Tsuchiya: Enzymatic Synthesis of Dextran, *Jour.* **63**: 293–295 (1952).

———, ———, N. N. Hellman, A. Kazenko, C. A. Hoffman, E. S. Sharpe, and Jackson: Enzyme Synthesis of Dextran, Acceptor Specificity and Chain tion, *Jour. Biol. Chem.*, **200**: 793–801 (1953).

Lockwood, A. R., and G. Swift: U.S. Patent 2,565,507, Aug. 28, 1951.

Lohmar, R.: Evidence of New Linkages in Dextrans, *Jour. Am. Chem. So* 4974 (1952).

McCleskey, C. S., L. W. Faville, and R. O. Barnett: Characteristics of *Leuco mesenteroides* from Cane Juice, *Jour. Bact.*, **54**: 697–708 (1947).

Owen, W. L., Jr., and W. L. Owen: Production of Gum Dextran, U.S. 2,392,258, Jan. 1, 1946.

Rankin, J. C., and A. Jeanes: Evaluation of the Periodate Method for Stru Analysis of Dextrans, *Jour. Am. Chem. Soc.*, **76**: 4435–4439 (1954).

Scott, T. A., Jr., and E. H. Melvin: Determination of Dextrin with Anthrone, *Chem.*, **25**: 1656–1661 (1953).

Senti, F. R., and N. N. Hellman: *Abstract Papers, Am. Chem. Soc.*, **121**: 80 (

Sloan, J. W., B. H. Alexander, R. L. Lohmar, I. A. Wolff, and C. E. Rist: Deter tion of Dextran Structure by Periodate Oxidation Techniques, *Jour. Am. Soc.*, **76**: 4429–4434 (1954).

Sohns, V. E., S. P. Rogovin, H. F. Conway, and C. T. Langford: Pilot-plant Pr tion of Clinical-sized Dextran by Acid Hydrolysis of the Enzymatically S sized High Polymer, *U.S. Dept. Agr., Bur. Agr. and Ind. Chem., Mimeogr Circ. Ser., AIC* 372, June, 1954.

Stacey, M.: Enzymic Production of Bacterial Polysaccharides, *Nature*, **149** (1942).

and G. Swift: Structure of Dextran Synthesized from Sucrose by a New ain of *Betacoccus arabinosaceous*, *Jour. Chem. Soc.*, 1948, p. 1555.

. G. L.: Method of Making Dextran, U.S. Patent 2,310,263, Feb. 9, 1943.

. F. H., H. J. Koepsell, and E. S. Sharpe: A New Disaccharide Produced by *uconostoc mesenteroides*, *Jour. Am. Chem. Soc.*, **74**: 3202 (1952).

ya, H. M., N. N. Hellman, and H. J. Koepsell: Factors Affecting Molecular ight of Enzymatically Synthesized Dextran, *Jour. Am. Chem. Soc.*, **75**: 757 953).

———, ———, J. Corman, C. S. Stringer, S. P. Rogovin, M. O. Bogard, Bryant, V. H. Feger, C. A. Hoffman, F. R. Senti, and R. W. Jackson: Factors ecting Molecular Weight of Enzymatically Synthesized Dextran, *Jour. Am. em. Soc.*, **77**: 2412–2419 (1955).

A. Jeanes, H. M. Bricker, and C. A. Wilham: Dextran-degrading Enzymes m Molds, *Jour. Bact.*, **64**: 513–519 (1952).

H. J. Koepsell, J. Corman, G. Bryant, M. O. Bogard, V. H. Feger, and R. W. kson: The Effect of Certain Cultural Factors on Production of Dextransu- se by *Leuconostoc mesenteroides*, *Jour. Bact.*, **64**: 521–526 (1952).

leve, J. W., W. C. Schaefer, and C. E. Rist: The Structure of NRRL B-512 xtran, *Jour. Am. Chem. Soc.*, **78**: 4435 (1956).

. P. R., and I. A. Wolff: Depolymerization of a Dextran with Sonic Vibra- ns or Ultra violet Light, *Jour. Am. Chem. Soc.*, **77**: 196 (1955).

ide-Carlson, V., and W. W. Carlson: The Vitamin Requirements of *Leuconos-* for Dextran Synthesis, *Jour. Bact.*, **58**: 135–141 (1949).

and ———: Studies on the Effect of Para-aminobenzoic Acid, Folic Acid, and fanilamide on Dextran Synthesis by *Leuconostoc*, *Jour. Bact.*, **58**: 143–149 49).

I. A., C. L. Mehltretter, R. L. Mellies, P. R. Watson, B. T. Hofreiter, P. L. trick, and C. E. Rist: Production of Clinical-type Dextran, Partial Hydrolytic polymerization and Fractionation of the Dextran from *Leuconostoc mesen- oides* Strain NRRL B-512, *Ind. Eng. Chem.*, **46**: 370–377 (1954).

R. L. Mellies, R. L. Lohmar, N. N. Hellman, S. P. Rogovin, P. R. Watson, W. Sloan, B. T. Hofreiter, B. E. Fisher, and C. E. Rist: Production of Clinical- e Dextran, Partial Acid Hydrolysis and Fractionation from *L. mesenteroides* RL B-742 and *S. dextranicum* NRRL B-1254, *Ind. Eng. Chem.*, **46**: 2605–2610 54).

———, and C. E. Rist: Fractionation of Dextran Products, U.S. Patent 2,007, June 28, 1955.

P. R. Watson, J. W. Sloan, and C. E. Rist: Controlled Thermal Depolymeri- on of Dextran, *Ind. Eng. Chem.*, **45**: 755–759 (1953).

## Some Patents concerning Dextran

A. E.: Plasticized Benzyl Dextran and Filament Formed Therefrom, U.S. ent 2,789,915, Apr. 23, 1957.

. J., and H. M. Tsuchiya: Enzymic Production of Dextrans of Intermediate ights, U.S. Patent 2,776,925, Jan. 8, 1957.

n, G. L.: Dextran Filament Manufacture, U.S. Patent 2,702,231, Feb. 15, 5.

M. J.: Dextran Solutions, U.S. Patent 2,787,518, Apr. 2, 1957.

L. J.: Food Preservation, U.S. Patent 2,790,720, Apr. 30, 1957.

and J. T. Tyree: Dextran Compound Fatty Acid Ester Lipstick, U.S. Patent 9,276, June 5, 1956.

R. A.: Production of Clinical Dextran, U.S. Patent 2,717,853, Sept. 13, 1955.

H. E.: Clinical Dextran Composition, U.S. Patent 2,708,174, May 10, 1955.

. H. A.: Icing Compositions Comprising a Carboxymethyl Dextran and hod of Producing the Same, U.S. Patent 2,789,911, Apr. 23, 1957.

Method of Preserving Food Products, U.S. Patent 2,790,721, Apr. 30, 1957.

. H. A., Jr.: Dextran Compound Coated Body Powder, U.S. Patent 2,749,277, e 5, 1956.

———: Dextran Coating Composition, U.S. Patent 2,734,828, Feb. 14, 1956.

———: Method of Preserving Shrimp, U.S. Patent 2,758,930, Apr. 14, 1956.

Wieman, R. L.: Preparing Clinical Dextran, U.S. Patent 2,784,143, Mar. 5, 19

Wimmer, E. L., and G. D. Lakata: Dextran, U.S. Patent 2,775,540, Dec. 25, 1

Wolff, I. A., R. A. Mellies, and C. E. Rist: Fractionation of Dextran Produ Patent 2,712,007, June 28, 1955.

———, P. R. Watson, and C. E. Rist: Thermal Modification of Dextran, U.S. 2,719,147, Sept. 27, 1955.

CHAPTER 21

# CHEESE

ese was prepared in Asia and in Europe several hundred years, at before the birth of Christ. A study of the origin of some of the different types of cheeses lies outside the scope of this text, but the sted reader will derive considerable enjoyment and add to his knowl- y perusing the history of cheese making.

**inition.** Cheese, according to the definition and standards of the and Drug Administration of the U.S. Department of Agricul- is:

> e product made from the separated curd obtained by coagulating the of milk, skimmed milk, or milk enriched with cream. The coagulation is lished by means of rennet or other suitable enzyme, lactic fermentation, a combination of the two. The curd may be modified by heat, pressure, g ferments, special molds, or suitable seasoning.
>
> name "cheese" unqualified means Cheddar cheese (American cheese, an cheddar cheese).

**ese Varieties.** More than 400 cheeses have been described by s (1953); however, he felt that there were only about 18 distinct brick, Camembert, cheddar, cottage, cream, Edam, Gouda, hand, rger, Neufchâtel, Parmesan, Provolone, Romano, Roquefort, Sap- wiss, Trappist, and whey cheeses.

**Milk.** Milk from ewes (sheep), goats, cows, mares, and other s has been used for the making of cheeses. Each of these milks con- ats, protein, milk sugar (lactose), mineral salts, and water. Fat ent as an emulsion, but the sugar, minerals, and some of the pro- re soluble in the water of milk. Casein (the principal protein) ined with calcium and exists in a colloidal condition.

**nation of Curd.** If acid is produced in milk as a result of the tation of lactose by bacteria, the casein is freed from calcium and ger appears as fine particles distributed throughout the medium, stead goes out of solution and eventually accumulates in large of curd or as a continuous mass.

et, an enzyme, likewise has the ability to alter the colloidal con- f casein, causing coagulation or curdling. This enzyme, sometimes as "rennin," is obtained commercially by extracting the fourth

---

*ce and Regulatory Announcements, Food Drug Admin.,* No. 2, Revision 5, 1936.

stomach of the calf, where it is principally found. Commercial sometimes serves as a successful substitute for rennet.

**Whey.** Whey is the watery portion of milk separated from th during cheese making. The principal constituents of whey are l soluble ash, and lactalbumin. A small amount of fats is a normal c nent of whey also.

**Treatment of Curd.** The curd formed in milk through the act rennet, lactic acid, or a combination of the two may be treate variety of manners. After it has settled into a fairly compact jel mass, it is usually cut into small pieces by special knives to fac drainage or removal of occluded whey. It may be drained with or out eventual pressure, depending on the type of cheese being ma tured. The curds of the soft or semisoft cheeses are pressed just suffic to remove the excess of whey, while those of the hard cheeses m heated and pressed in a form with weights until the curd forms a mat. The curd is treated with varying quantities of salt by va methods. Finally the treated curd is molded into the desired shap is ready for ripening or curing.

**Ripening.** During this process, the flavor, composition, and ph properties of the cheese undergo change owing to the action of chara istic molds or bacteria and their enzymes. The nature of the ch depends on the method used in treating the curd, the method and qua of salting, the microorganisms present or added, and the control of perature and relative humidity in the curing rooms, chambers, or cav

**Classification.** Cheeses may be classified in several manners: on basis of hardness, as hard, semihard, and soft cheeses; on the basis o

TABLE 110. A CLASSIFICATION OF CHEESES

| Name of cheese | Place of origin | Name of cheese | Place of o |
|---|---|---|---|
| Hard rennet cheeses | | Semihard rennet cheeses | |
| Cheddar | England | Limburger | Belgium |
| Cheshire (Cheddar type) | Chester, England | Stilton (mold) | Stilton, Engl |
| | | Brie (mold) | France |
| Caciocavallo | Southern Italy | Blue (mold) | France, Denm etc. |
| Gorgonzola (mold) | Italy (near Gorgonzola) | Camembert (mold) | France |
| Parmesan | Italy | Roquefort (mold) | France |
| Pecorino | Italy | Münster | Near Münste Germany |
| Provolone | Italy | | |
| Reggiano | Italy | Brick | United State |
| Romano | Italy | | |
| Edam | Edam, Holland | Soft rennet cheeses | |
| Gjedost | Norway | Port du Salut | Canada |
| Emmenthaler | Switzerland | Neufchâtel | France |
| Gruyère (Swiss) | Gruyère, Switzerland | Soft lactic cheeses | |
| Pineapple | United States | Cottage | |
| | | Cream | United States |

pal ripening agents, as mold or bacterial cheeses; on the basis of nd of milk from which manufactured, as cow, ewe, goat, or other on the basis of the country of origin, as Italian, French, American, e like; on the basis of the method of coagulation, as rennet or acid heese; on the basis of added substances, as sage; or on the basis fat content of the milk, as full-cream, whole-milk, or skim-milk s.

Table 110 cheeses are classified as hard, semihard, and soft rennet s, and as lactic cheeses. Sometimes Roquefort and Stilton cheeses ssified as hard cheeses and Brick, Brie, Camembert, Limburger, ünster as soft cheeses, rather than as semihard cheeses. Blue . Brie, Camembert, Gorgonzola, Roquefort, and Stilton are characl as mold cheeses.

**lyses of Some Varieties of Cheese.** Table 111 contains the averalyses of some varieties of cheeses. Analyses of additional varieties e found in the publication by Sanders (1953).

**ddar Cheese.** Cheddar cheese, a hard cheese prepared from whole y the action of rennet, originated in Cheddar, England. It is the g cheese manufactured in the United States and is widely made by glish.

milk used for the manufacture of Cheddar cheese is analyzed, rdized, weighed, and heated to about 86°F. (30°C.), and a suitable of lactic acid bacteria is added. Vegetable coloring matter may rporated also at this time if it is to be used. The milk is thoroughly and when the acidity is correct (0.17 to 0.2 per cent) rennet ximately 3 oz. for each 1,000 lb. of milk), diluted with water, is It requires only 20 to 40 min. to coagulate the casein. When tion is satisfactory, demonstrated by a clean breaking of the curd, a clear whey, the curd is cut by special knives into cubes about square to permit the whey to escape more readily. The curd is heated to about 100°F. (37.8°C.). Acidity and body are devel- the curd as it is cooked with gentle agitation to keep it from . The whey is drained off when the curd has assumed the desired

curd is now cut into large pieces, which are turned frequently ced one on top of the other to force out the whey. This treatment n as cheddaring.

dared curd is then cut up and milled into small pieces by special es. Salt, at the rate of approximately 1.5 lb. to each 1,000 lb. of added and carefully mixed with the curd. The product is cooled . (26.7°C.), packed in hoops containing cheesecloth linings, and for several hours to the desired shape.

ng the ripening process, which was originally carried out in the r caves and which may require a few weeks or several months, eria and enzymes present bring about characteristic changes in the Lactic acid bacteria, such as *Streptococcus lactis* and *Lactobacillus* re especially important in the ripening or curing of the Cheddar The bacteria increase enormously, often reaching several hundred

TABLE 111. AVERAGE ANALYSES OF SOME VARIETIES OF CHEESE *

| Variety | Authority | Water, per cent | Fat, per cent | Proteides, amides, etc., per cent | Milk sugar, lactic acid, etc., per cent |
|---|---|---|---|---|---|
| Brick | Bureau of Chemistry | 42.47 | 30.66 | 21.05 | .... |
| Brie: | | | | | |
| Imported | Bureau of Chemistry | 52.53 | 22.44 | 20.94 | .... |
| American | Bureau of Chemistry | 52.10 | 24.72 | 19.60 | .... |
| Caciocavallo | Bureau of Chemistry | 34.95 | 21.98 | 34.33 | .... |
| Camembert, imported | Bureau of Chemistry | 47.88 | 26.32 | 22.21 | .... |
| Cheddar skim | Bureau of Chemistry | 57.04 | 4.88 | 32.09 | .... |
| Cream-French, Demi-sel | Bureau of Chemistry | 42.74 | 39.88 | 14.49 | .... |
| Edam (American) | Bureau of Chemistry | 38.07 | 22.65 | 30.89 | .... |
| Emmenthaler | Lindt | 33.00 | 30.50 | 30.44 | .... |
| Gorgonzola | Musso | 37.30 | 34.67 | 25.16 | 1.62 |
| Gouda | Cribb | 54.79 | 9.02 | 25.94 | .... |
| Gruyère | Balland | 29.99 | 28.19 | 33.03 | 4.82 |
| Limburger: | | | | | |
| American | Arnold | 35.64 | 29.82 | 28.53 | .... |
| Imported | Bureau of Chemistry | 54.79 | 19.61 | 21.27 | .... |
| Münster (American) | Bureau of Chemistry | 40.60 | 31.00 | 22.20 | .... |
| Neufchâtel | Bureau of Chemistry | 52.05 | 23.51 | 19.33 | .... |
| American | Bureau of Chemistry | 59.22 | 18.17 | 21.30 | .... |
| Parmesan: | | | | | |
| Formaggio | Bureau of Chemistry | 16.95 | 22.71 | 49.39 | .... |
| Reggiano | Bureau of Chemistry | 29.63 | 27.29 | 34.84 | .... |
| Pecorino | Sartori | 29.80 | 30.51 | 33.51 | .... |
| Romano | Bureau of Chemistry | 29.56 | 27.69 | 31.20 | .... |
| Pineapple | Johnson | 24.07 | 38.12 | 29.35 | 2.49 |
| Port du Salut | Bureau of Chemistry | 50.10 | 25.17 | 21.18 | .... |
| Roquefort | Currie | 38.69 | 32.31 | 21.39 | .... |
| Stilton | Bureau of Chemistry | 35.57 | 31.19 | 28.96 | .... |
| Swiss: | | | | | |
| American | Bureau of Chemistry | 34.28 | 32.60 | 27.55 | .... |
| Imported | Bureau of Chemistry | 33.91 | 30.61 | 29.22 | .... |

* Data tabulated from *U.S. Dept. Agr., Bull.* 608, rev. February, 1932.

millions in numbers per gram during the first few weeks, and then gra
ally die off until only a few million per gram remain at the end
8 months.

It is necessary to prevent molds from growing on this cheese, for th
cause a rapid deterioration of the product. This is done by use of s
on the surface.

A yield of approximately 1 lb. of cheese is obtained from 10 lb. of m
by this process.

**uefort Cheese.** Roquefort cheese is a semihard, friable cheese
terized by a mottled or marbled appearance of the interior, owing
presence of *Penicillium roqueforti*, a blue-green mold. This cheese
en known for almost a thousand years, having originated in the
of France. It is made principally from the milk of ewes, bred par-
ly for their high milk-producing abilities. The sheep graze in the
region of the Cevennes. Their milk is made into cheese in small
es distributed over this relatively small section of Southern France.
eese is transported to the vicinity of Roquefort for ripening.

aking Roquefort cheese, the curd is drained in hoops but not
l. It is inoculated at this time with a dried bread product rich in
pores, the inoculum being prepared by growing *P. roqueforti* in the
r of bread in a moist, cool place until the bread becomes a moldy
ontaining a vast number of spores. The bread is dried carefully
ound to a powder, the latter being used as the inoculum.

ng ripening, the temperature and relative humidity must be care-
ontrolled in order to obtain a fine cheese. The temperature of ripen-
st not be much higher than 48°F. (8.9°C.), and the relative
ty should be high. In France, the ripening is carried out in natural
nd in artificial caves hewn from the limestone. Water trickling
hrough crevices cools the air and at the same time nearly saturates
flow of air through the caves is regulated in order to obtain the
temperature and humidity. In the United States, Roquefort-type
is made in curing rooms having regulated relative humidity and
ature; in caves; and, in one instance at least, in a mine shaft.

s are punched into the curd to facilitate the development of the
hroughout the cheese. Although aerobic, *P. roqueforti* will grow
minimum supply of air. After the mold has developed to the
extent, the cheese is wrapped in tin foil and stored at approxi-
40°F. (4.44°C.), at which temperature the enzymes of the mold
ve but the mold growth is inhibited.

ungent taste of Roquefort is due in part to the action of lipase,
litting enzyme, which liberates caproic, caprylic, and capric acids
e fat.

**-Cheese.** A great deal of cheese, made by a process similar to that
efort but from cow's milk, is fabricated and consumed in this
and elsewhere. Such cheese is commonly known as "blue cheese."
ufacture has been described by Babel (1953).

**s Cheese.** One of the most difficult varieties of cheese to make is
heese. Three different starters are used in its preparation: *Strept.*
*hilus, L. bulgaricus* or *L. lactis*, and *Propionibacterium shermanii*
e-producing organism). The making of Swiss cheese has been
d by Sanders, Walter, and Tittsler (1955).

**iptions of Cheese Varieties.** Considerable information on cheese
will be found in the publications listed at the end of this

tions and standards of identity of cheese, processed cheeses,
ods, cheese spreads, and related foods may be found in Food and

Drug Administration *Service and Regulatory Announcements,* Title 21, Chap. I, Part 19.

### Some References concerning Cheeses

Babel, F. J.: The Role of Fungi in Cheese Ripening, *Econ. Botany,* 7(1 (1953).

Davis, J. G.: "A Dictionary of Dairying," Leonard Hill, Ltd., London, 1950.

——— and F. J. MacDonald: "Richmond's Dairy Chemistry," 5th ed., Griffin and Co., Ltd., London, 1957.

Hammer, B. W., and F. J. Babel: "Dairy Bacteriology," 4th ed., John Wiley Inc., New York, 1957.

Peter, A., und E. Zollikofer: "Lehrbuch der Emmentalerkäserei," 8th ed., K. Erben A. G., Bern, 1942.

Sanders, G. P.: Cheese Varieties and Descriptions, *U.S. Dept. Agr., Agr. H* 54, 1953.

———, H. E. Walter, and R. P. Tittsler: General Procedure for Manufacturi Cheese, *U.S. Dept. Agr., Circ.* 851, August, 1955.

U.S. Food and Drug Administration: Cheeses and Cheese Products. Definiti Standards under the Federal Food, Drug and Cosmetic Act, *U.S. De Service and Regulatory Announcements, Food Drug Admin.,* No. 2, 1952 (with amendments).

Van Slyke, L. L., and W. V. Price: "Cheese," Orange Judd Publishing Compa New York, 1952.

## CHAPTER 22

# PRODUCTION AND PROPERTIES OF 2,3-BUTANEDIOL

Butanediol ($CH_3 \cdot CHOH \cdot CHOH \cdot CH_3$) is well known as 2,3- ne glycol. It is somewhat less commonly known as 2,3-dihydroxy- or dimethylethylene glycol.

demand for synthetic rubber in World War II greatly stimulated h on the production and properties of 2,3-butanediol in this country Canada, since it could be converted to 1,3-butadiene, a substance ı the production of rubber of the buna type. Since insufficient dge was available concerning the production of 2,3-butanediol and sion methods at the time the synthetic rubber industry was being shed, it was necessary to manufacture butadiene from other raw ıls, such as ethyl alcohol. During the war years and since, a great information has been obtained regarding production methods, larly on laboratory and pilot-plant scales. In addition, it has ıown that derivatives may be readily prepared from the 2,3- liols and that there are many potential uses for these, such as for eze agents, solvents, softeners, and plastics.

**rical.** The literature on this subject has been reviewed by Led- . Adams, and Stanier (1945), and by Underkofler and Fulmer

first significant reports concerning the *Aerobacter* fermentation ›se of Harden and Walpole (1906); Walpole (1911); and Scheffer Following these there appeared a number of publications from a State College, among which may be mentioned those of Breden Breden and Fulmer (1931); Fulmer, Christensen, and Kendall Kendall (1934); Chappell (1935); Porter, McCleskey, and Levine Silverman and Werkman (1941); Stahly and Werkman (1942); lerkofler and Fulmer (1948).

rts concerning the fermentation brought about by *Aerobacillus xa*[1] have issued largely from Canada, although the first significant tion regarding this fermentation was supplied by Donker (1926) ınd, who showed that 25 per cent of 2,3-butanediol and 19 per cent ıol were obtained in the fermentation of glucose. A large number ts have been published since 1944, in particular by Ledingham, dams, Stanier, Rose, Leslie, Fratkin, and their associates of the

---

[1] rganism is classified as *Bacillus polymyxa* in the seventh edition of "Bergey's f Determinative Bacteriology" (The Williams & Wilkins Company, Balti- 7).

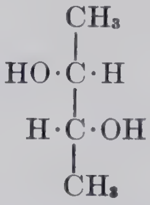
National Research Council of Canada; and also by Katznelson, Lo and fellow workers of the Division of Bacteriology and Dairy R Canada Department of Agriculture.

During World War II, a considerable amount of coordinated r was carried out simultaneously by personnel of the Northern R Research Laboratory of the U.S. Department of Agriculture; the N Research Council and the Department of Agriculture in Canad Iowa State College; the University of Wisconsin; Joseph E. Seagra Sons, Inc.; the Commercial Solvents Corporation; and Schenley D Corp.; and by members of other laboratories. In the following para reference is made to the results of some of the research carried these organizations and other individuals.

**The Fermentations.** A number of species or strains of bacteria fied in the genera *Aerobacter, Aerobacillus, Aeromonas, Serrati Bacillus* possess the ability to produce 2,3-butanediol. The fermen brought about by strains of *Aerobacter aerogenes* and *Aerobacillus myxa* (Prazmowski) Migula have been studied intensively and to be most important ones. However, considerable interest has been in the fermentations produced by *Aeromonas hydrophila, Bacillus s* (Ford's strain), and *Serratia marcescens*.

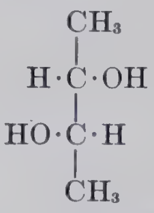
**Products of the Fermentations.** The products formed during type of fermentation vary qualitatively and quantitatively, depend the strain of organism used, the media, and the conditions of ferment However, in each instance, there are produced 2,3-butanediol, a (acetylmethylcarbinol), ethanol, carbon dioxide, acids, and occasi other substances. These will be considered in detail in the foll discussion.

**Forms of 2,3-Butanediol.** There are three stereoisomeric for 2,3-butanediol, or 2,3-butylene glycol, all of which are produce bacterial fermentation. These are the dextro, levo, and meso form structural formulas of which are shown below:



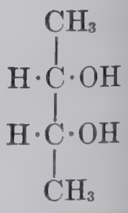
$$\begin{array}{c} CH_3 \\ | \\ HO\cdot C\cdot H \\ | \\ H\cdot C\cdot OH \\ | \\ CH_3 \end{array}$$

D(−)-2,3-Butanediol
(Levorotatory form)



$$\begin{array}{c} CH_3 \\ | \\ H\cdot C\cdot OH \\ | \\ HO\cdot C\cdot H \\ | \\ CH_3 \end{array}$$

L(+)-2,3-Butanediol
(Dextrorotatory form)



$$\begin{array}{c} CH_3 \\ | \\ H\cdot C\cdot OH \\ | \\ H\cdot C\cdot OH \\ | \\ CH_3 \end{array}$$

*meso*-2,3-Butanediol
(Optically inactive form)

A mixture of the dextrorotatory and meso forms of 2,3-butaned produced by strains of *A. aerogenes*.

The levorotatory form of 2,3-butanediol is produced characteristi by *B. polymyxa* (Prazmowski) Migula. Ward and his associates (1 identified D(−)-2,3-butanediol as the chief substance formed by *B. myxa* in grain mashes. This substance possessed a specific rot slightly in excess of −13.0°. According to Neish (1945), the D(− butanediol produced by *B. polymyxa* is a pure isomer, with the o rotation $[\alpha]^{26}_{D} = -13.34°$.

ixture of the levorotatory and meso forms of 2,3-butanediol is ed by *A. hydrophila*. According to Stanier and Adams (1944) vorotatory form preponderates, based on the optical rotation = −0.97.

*ubtilis* (Ford's strain) produces a mixture of the levorotatory and orms of 2,3-butanediol in approximately equal quantities, accord- Neish, Blackwood, and Ledingham (1945*a*, *b*). The optical rotation quals −5.0°. The refractive index is 1.4340 at 26°C., while the point is 181 to 182°C. at 760-mm. pressure.

2,3-butanediol produced in the *S. marcescens* fermentation is a e of the meso and dextrorotatory forms (Neish et al., 1947), but ount of the dextrorotatory form is only about 2 per cent of the

**luction of 2,3-Butanediol.** As already indicated, 2,3-butanediol uced in reasonable yields by strains of several different groups of a. These will be considered individually since production methods

*he* A. aerogenes *Fermentation.* This fermentation was first studied rden and Walpole in 1906. The organism *B. lactis aerogenes* (*A. nes*) was grown under anaerobic conditions on a medium containing ent Witte peptone, 2 per cent glucose (or mannitol), and chalk. (1911) Walpole investigated the fermentation further. In 1928, r carried out research on the production of 2,3-butanediol by *genes* and related organisms.

er, Christensen, and Kendall (1933) investigated the effect of concentration on the production of 2,3-butanediol by species of *cter*, particularly *A. pectinovorum* (a variety of *A. aerogenes*). dium contained the following materials per 100 ml.: 0.250 g. of ium chloride, 0.150 g. of potassium monophosphate, 0.150 g. of chloride, and 0.20 g. of magnesium sulfate, plus different quanti- sucrose. The pH was adjusted to an optimum of 6 with sodium te. Sucrose in concentrations up to and including 8 per cent was fermented. As the concentrations were increased above 8 per e per cent of sucrose fermented dropped. At a concentration of nt, 47 g. of 2,3-butanediol were produced from 100 g. of sucrose .

ODUCTION FROM ACID-HYDROLYZED STARCHES. Ward, Pettijohn, ghill (1945) have studied the production of 2,3-butanediol from drolyzed corn and wheat starches by *A. aerogenes* NRRL B-199. h was carried out on the starches since it was believed that 2,3- liol could be recovered more readily from fermented mashes d from them than from fermented mashes prepared from whole

cultures of *A. aerogenes* were carried on slants of agar (contain- of Difco tryptone, 1 g. of glucose, 5 g. of Difco yeast extract, and agar per liter).

tarter (inoculum) for the fermentation mashes was prepared by *A. aerogenes* in a special medium composed of the following

ingredients to which was added subsequently separately sterilized
cent urea solution:

| | G. |
|---|---|
| Glucose | 50 |
| $KH_2PO_4$ | 0.60 |
| $MgSO_4\cdot 7H_2O$ | 0.25 |
| $CaCO_3$ | 5.0 |
| Water, distilled, to make 1 liter | |

The above medium was distributed into 200-ml. Erlenmeyer fl
100-ml. amounts and sterilized with steam at a pressure of about
per sq. in. (121°C.) for 30 min. At the time of inoculation eac
received 1 ml. of sterile 20 per cent urea solution.

The fermentation mashes were prepared by cooking with steam
pressure, suspensions of starch in water that had been acidulate
hydrochloric or sulfuric acids, and by adding the necessary sal
calcium carbonate. The concentration of starch used was generally
10 per cent. Sufficient chemically pure acid was added to the
slurries to yield an acid concentration of 0.02 to 0.12 normality.
pressure-cooking the mashes, the starch slurries were gelatinized i
water baths while being agitated. They were then cooked for 1
4 hr. at a steam pressure of 22 to 25 lb. per sq. in. (128 to 131°C.),
which time the starches were saccharified. Table 112 shows the ef

TABLE 112. EFFECT OF COOKING CONDITIONS ON BUTANEDIOL YIELD FROM STARCH

(Mashes 1 and 2 hydrolyzed 1.25 hr. at 130°C.; mashes 3 and 4 hydrolyzed
130°C.; distilled water used throughout)

| Fermentor no. | HCl concentration | Sugar, g./100 ml. Original Free | Sugar, g./100 ml. Original Total | Sugar, g./100 ml. Final Free | Fermentation time, hr. | Products, g./100 ml. Butanediol | Products, g./100 ml. Acetoin |
|---|---|---|---|---|---|---|---|
| 1 | 0.12 *N* | 10.04 | 10.36 | 0.30 | 32 | 3.72 | 0.20 |
| 2 | 0.12 *N* | 10.35 | 10.36 | 0.38 | 33 | 3.74 | 0.11 |
| 3 | 0.04 *N* | 10.25 | 10.30 | 0.52 | 47 | 4.23 | 0.21 |
| 4 | 0.02 *N* | 10.22 | 10.48 | 0.49 | 40 | 4.18 | 0.16 |

* G. E. Ward, O. G. Pettijohn, and R. D. Coghill, *Ind. Eng. Chem.*, 37
(1945). Copyright 1945 by the American Chemical Society and reprinted by p
sion of the copyright owners.

cooking conditions on yields of 2,3-butanediol from starch 2778, a re
wheat starch containing 8.19 per cent moisture and 98 per cent star
a dry basis.

Salts to supply the nutritive requirements of *A. aerogenes* were a
to the starches in the following amounts per liter: 0.25 g. of $MgSO_4\cdot 7$
0.60 g. of $KH_2PO_4$, 2 g. of urea, and 5 to 10 g. of $CaCO_3$. The magne
sulfate and potassium dihydrogen phosphate were added to the s

s before cooking. The urea (as a 20 per cent solution) and calcium ıate (dry) were sterilized separately and added to the saccharified es before inoculation. The pH was adjusted to 5.5 to 6 by means added $CaCO_3$.

fermentations were carried out in rotary drums of the type de- l on page 580 under the following conditions: temperature = 30°C., tions per minute = 10 to 12, gauge pressure = 5 lb., air flow = 50 ml. per min. They were concluded in each case when tests showed he minimum reducing sugar concentration was reached, which led with the maximum production of 2,3-butanediol.

yields of 2,3-butanediol, acetoin, and ethyl alcohol obtained from ydrolyzates of starch 2778, as calculated in terms of pounds per of starch and per 34 lb. of pure dry starch (the equivalent of 1 bu. ı), and of per cent of theoretical on the basis of the glucose con- are shown in Table 113.

TABLE 113. YIELDS FROM STARCH 2778 *

| Yield, lb. 100 lb. starch (as received) | | | Yield, lb. 34 lb. pure dry starch | | | Yield, per cent of theoretical, based on glucose consumed † | | |
|---|---|---|---|---|---|---|---|---|
| Butanediol | Acetoin | Ethyl alcohol | Butanediol | Acetoin | Ethyl alcohol | Butanediol | Acetoin | Ethyl alcohol |
| 32.5 | 1.8 | 2.9 | 12.3 | 0.7 | 1.1 | 75 | 4 | 7 |
| 32.7 | 1.0 | 2.2 | 12.4 | 0.4 | 0.8 | 74 | 2 | 5 |
| 36.8 | 1.9 | 0.6 | 13.9 | 0.7 | 0.2 | 86 | 4 | 1 |
| 36.4 | 1.4 | 1.8 | 13.8 | 0.5 | 0.7 | 85 | 3 | 4 |

. Ward, O. G. Pettijohn, and R. Coghill, *Ind. Eng. Chem.*, 37: 1189 (1945). ıt 1945 by the American Chemical Society and reprinted by permission of right owners.

'ose consumed" is assumed to equal original free sugar minus final free

ıy be concluded from the researches of Ward and his coworkers drolyzed starch mashes properly balanced in respect to ions, per- the use of an ion-exchange agent, and supplied with the usual materials, may be fermented under optimum conditions by *genes* with the production of satisfactory yields of 2,3-butanediol. er, higher yields on the average may be obtained from acid- zed whole-grain mashes which do not require the same degree of ıent.

ODUCTION FROM WOOD HYDROLYZATES. Perlman (1944) has investi- ıe production of 2,3-butanediol from acid hydrolyzates of hard woods, including southern red oak, Douglas fir, white spruce, and ı yellow pine.

ODUCTION FROM GLUCOSE. Olson and Johnson (1948) described ıuction of 2,3-butanediol from glucose. Under optimum conditions wherein aeration was used, yields of 80 mM. of 2,3-butanediol obtained per 100 mM. of glucose with *A. aerogenes* 199. Sugar in co trations up to 10 per cent was fermented in less than 48 hr. Su concentrations up to 26.5 per cent was fermented in 108 hr. when fed slowly as a concentrated substrate during the course of the ferm tion. In fermentations such as the latter, the concentration of obtained was as high as 98 g. per liter.

2. *The* B. polymyxa *Fermentation.* This fermentation is charact by the production of the levorotatory form of 2,3-butanediol and et from mashes containing saccharified ground whole grains, such as w

Some of the first contributions concerning the fermentation were nished by Donker (1926), who studied carbohydrate dissimilatio *Aerobacillus polymyxa* (Prazmowski) Donker. Later Kluyver and S fer (1933) investigated the fermentation. During the years 1942–1 very large amount of intensive research was carried out on this ferm tion, particularly by Ledingham, Adams, Stanier, and their associat the Canadian National Research Laboratories, and Katznelson and L head of the Canadian Department of Agriculture.

The fermentation may be conveniently discussed under the follo headings: the organism, the inoculum, and other factors affecting fermentation.

THE ORGANISM. The fermentation is carried on by *B. polymyxa* (P mowski) Migula. There are other synonyms for this species, suc *A. polymyxa* (Prazmowski) Donker, *Clostridium polymyxa* (Prazm ski), *Granulobacter polymyxa* (Beijerinck), and *B. asterosporus* Meyer) Migula.

The organism has been described by Donker (1926), by Po McCleskey, and Levine (1937), by Ledingham, Adams, and Sta (1945), and by others.

*B. polymyxa* is a sporeforming, Gram-negative, facultative, aer organism of the *Aerobacillus* Donker group and family Bacillaceae. motile with peritrichous flagella and occurs vegetatively as rods vary in size from about 0.5 to 1 μ in width to 2 to 8 μ in length liquefies gelatin, produces catalase, hydrolyzes starches, and giv positive Voges-Proskauer reaction. Most strains ferment arabin cellobiose, dextrins, galactose, glucose, inulin, lactose, levulose, mann mannose, raffinose, salicin, sorbitol, starch, trehalose, and xylose. S but not all, strains ferment rhamnose. The optimum temperature app to be about 30°C.

Strains of *B. polymyxa* may be isolated from a wide number of sou including soil, water, milk, grains, feces, and fresh or decaying plants

Ledingham, Adams, and Stanier recommended the following proce for its isolation: Agitate some soil or other source material with st water. Place 5 ml. of the suspension thus prepared in a sterile cul tube and pasteurize it in a water bath at a temperature of 80°C. 10 min. Inoculate tubes containing sterile lactose or starch broth w 1-ml. portions of the pasteurized suspension and incubate the tu at 30°C. Streak plates containing neutral red, starch-peptone, or sta ıgar with material from the tubes showing gas production after 2 to Select and isolate typical colonies.

colonies of *B. polymyxa,* which show a wide range of variability ng to Ledingham and his associates, are generally pink or red in hen grown on the neutral red agar. They give rise to a pleasant, ke odor. Some, which form a thick slime, may become distended bubbles. Growth and spreading of the colonies on moist plates apidly.

14. The Composition of Some Media Used in Isolating *Bacillus polymyxa* *

| Starch broth, per cent | | Neutral red agar | |
|---|---|---|---|
| ........................ | 1 | Starch or lactose ............ | 20 g. |
| ........................ | 1.5 | Peptone .................... | 10 g. |
| Lactose broth, per cent | | Yeast extract ............... | 5 g. |
| | | Neutral red ................ | 0.05 g. |
| ........................ | 1 | Agar ...................... | 15 g. |
| ........................ | 1 | Water ..................... | 1,000 ml. |

obtained from article by G. A. Ledingham, G. A. Adams, and R. Y. *Can. Jour. Research,* **F, 23**: 48 (1945).

ıs of *B. polymyxa* should be selected primarily for their ability to high yields of 2,3-butanediol and ethanol, and also on the basis of rability of the mashes fermented by them. Stability of charac- is another factor of importance in the selection of the most strains for fermentation.

elson (1944) observed that there was a difference in the filter- f mashes, some filtering within a few minutes and others requir- rs. The difference in filterability was found to be a strain ristic.

r, Adams, and Ledingham studied strains of *B. polymyxa* in to the production of 2,3-butanediol and the filterability of the d mashes. They found that it is impossible to correlate colony nce with filterability and yields. Filtration of mashes fermented in strains, including both high and low producers of 2,3-butane- readily accomplished, while that of other strains was accom- vith great difficulty. Mashes were classified on the basis of their ity. A type A mash was one in which the residual solids of ation collected on the surface of the medium, leaving a rather uid below. The type B mash was one in which the residual ccumulated as a loose sediment with a relatively clear layer of ove. The type C mash was one in which the solids were held in niform suspension as a result of the large amount of loose slime by the organism. Both type A and type B mashes presented lties in respect to filtration, but the type C mash filtered very Strains producing the type C mash, even though good producers diol, are not considered to be practical for commercial use.

Stanier and his associates pointed out the value of preserving of *B. polymyxa* by drying in order to prevent further variations.

THE INOCULUM. The inoculum is the starter used to seed th fermentation medium. Particular attention must be paid to the se of an inoculation medium that will favor the rapid development bacteria and produce optimum yields of the desired end products respect to the use of the inoculum, it is necessary to know at wh it should be employed and in what quantity.

Ledingham, Adams, and Stanier used two types of inoculation which are listed below. Medium 1 has the advantage that it m pipetted more readily than medium 2.

| Medium 1 | Medium 2 |
|---|---|
| Soluble starch, 2% | Whole wheat, 5% |
| Yeast extract, 0.5% | Yeast extract, 0.5–1.0% |
| Calcium carbonate, 1% | Calcium carbonate, 1% |

The yeast extract used in these media may be substituted by malt sp corn-steep liquor, malt extract, or dried yeast.

Katznelson used, in addition to the above media, the Difco M Red–Voges-Proskauer medium.

The age of the inoculum, within the limits of 10 to 96 hr., appa makes little difference on the yield of 2,3-butanediol and ethyl a provided that the organism is active.

The size of the inoculum used (provided that it represents at least cent of the volume of the fermentation medium) is also not too influ on the yields. Katznelson found that an inoculum of 0.5 per cent siz too small but that the use of quantities representing 2.5 to 5 per ce the volume of the fermentation mash was satisfactory. Larger am may be used, of course, if desired.

Table 115 shows the influence of age of inoculum, quantity of i lum, and nature of the inoculating medium on yields of 2,3-butanedi a strain of *B. polymyxa*.

TABLE 115. INFLUENCE OF AGE AND QUANTITY OF INOCULUM AND INOCULATING M ON YIELD * OF BUTANEDIOL BY STRAIN 47

| Age of inoculum, hr. | 2,3-Butanediol, per cent | Amount of inoculum, per cent | 2,3-Butanediol, per cent | Nature of inoculating medium | 2,3-B di per |
|---|---|---|---|---|---|
| 24 | 3.16 | 0.5 | 2.60 | Yeast-starch-$CaCO_3$ | 3 |
| 48 | 3.21 | 2.5 | 3.04 | Difco M.R.–V.P. | 3 |
| 96 | 3.17 | 5.0 | 3.04 | 5% whole-wheat mash | 3 |

* H. Katznelson, *Can. Jour. Research,* **C**, 22: 235 (1944).

RAW MATERIALS USED. *B. polymyxa* is capable of fermenting a va of properly prepared grain mashes, such as corn and wheat; glu xylose; mannitol; and other carbohydrates. The utilization of co wheat appears desirable when these grains are available in large tities. The use of whole wheat has been studied extensively by igham and his associates and by Katznelson.

CENTRATION OF RAW MATERIALS. The choice of the optimum conation of raw material must be based on fermentation efficiency and viscosity. Ledingham and his coworkers found that mashes which ined more than 15 per cent of wheat by weight were inefficiently nted and were generally thick and viscous. Such mashes gave higher than those of lower concentrations, but the time required for fertion was prolonged and the starches were incompletely utilized. A ntration of whole wheat of approximately 15 per cent was considered Viscosity is a factor of importance especially in connection with ing or pumping of the mashes.

ECT OF PARTICLE SIZE OF WHEAT. The investigators at the Canadian rch Laboratories found that the yields of 2,3-butanediol and ethyl ol were not adversely affected by the particle size of wheat provided he kernels were broken.

PARATION OF THE MASH. The preparation of the mash includes the ing out and the mixing of the ingredients as well as its cooking or zation.

lingham and his collaborators placed 300-ml. portions of medium, preliminary treatment, into 500-ml. Erlenmeyer flasks. A 15 per nash thus contained 45 g. of wheat in 300 ml. of medium. To each 1 per cent of calcium carbonate was added, except when special were carried out to determine the effect of pH or carbonates. In to avoid the caking of starches in the mashes during sterilization, gredients were gelatinized by cooking lightly with constant agitand then placed in the Erlenmeyer flasks. Sterilization was ordiaccomplished by the use of steam at a pressure of 15 lb. (121°C.) r., a relationship established after experiment had been carried out ermine the effect of various combinations of temperature and time king on the production of 2,3-butanediol and ethanol. It was disd that cooking mashes for 3 hr. at temperatures of 126°C. or higher d in lowered yields. Table 116 indicates that the time and temperaf cooking may be varied considerably without detrimental effects yields.

ingham and his associates occasionally preliquefied their grain s by the addition of malt (1 per cent of the weight of the grain) and lding the temperature at 70°C. for 10 min. This facilitated the quent agitation of the mash and the uniform dispersion of the um.

znelson prepared mashes of the following composition in 1-liter neyer flasks: 17.8 g. of coarse whole-wheat flour, 1 g. of calcium nate, and 100 ml. of water. The concentration of wheat was thus cent, based on a total mash weight of 118.8 g. The mashes were l for 1 hr. with steam at a pressure of 15 lb. per sq. in. Katznelson d that the ratio of surface area to volume produced under these stances produced higher yields of 2,3-butanediol and ethyl alcohol

Table 116. Effect of Cooking Time and Temperature on 2,3-Butaned
Ethanol Production by *Bacillus polymyxa* C 3(2) after 72-hr. Ferment
on 15 Per Cent Mash *

| Time of cooking, hr. | Temperature, °C. | | | | |
|---|---|---|---|---|---|
| | 100 | 108.1 | 115.1 | 121.0 | 126.0 |
| | 2,3-Butanediol, per cent | | | | |
| ½ | 2.58 | 2.77 | 2.75 | 2.67 | 2.62 |
| 1 | 2.72 | 2.77 | 2.65 | 2.84 | 2.70 |
| 3 | 2.95 | 2.81 | 2.57 | 2.67 | 2.15 |
| | Ethanol, per cent | | | | |
| ½ | 1.71 | 1.74 | 1.72 | 1.68 | 1.67 |
| 1 | 1.77 | 1.77 | 1.69 | 1.64 | 1.65 |
| 3 | 1.86 | 1.69 | 1.61 | 1.47 | 1.14 |

* G. A. Ledingham, G. A. Adams, and R. Y. Stanier, *Can. Jour. Research,*
48 (1945).

than were obtained when larger amounts of mash were used in a fla the same size.

PH CONTROL. The optimum pH for the production of 2,3-butan and ethyl alcohol from whole-wheat mashes of approximately 15 per concentration by *B. polymyxa,* appears to lie within the range of 5.6 t

The pH of unbuffered wheat mashes lies close to neutrality (6 7), according to Ledingham and his associates, but drops to 5.5 during the fermentation unless a neutralizing agent is added to medium.

Although the pH of the fermentation mash may be controlle several different agents, the use of calcium carbonate or ammonia ap to produce particularly favorable results. In the laboratory, the use per cent calcium carbonate prevents the pH from falling below 5.6 to

Other carbonates, such as barium and magnesium, are not as effe as calcium carbonate when employed at the same concentration, acco to Ledingham, Adams, and Stanier. The use of magnesium carbo results in a reduction of the butanediol/ethanol ratio.

Adams and Leslie (1946) reported that the use of calcium carbonat a commercial scale may offer certain disadvantages. For example, stated that the pH cannot be maintained at a uniform level throug the fermentation period, that cooking mashes which contain calc carbonate has an adverse effect on the subsequent fermentation, and the presence of large amounts of calcium carbonate in the unferme residues lowers their food value for animals on account of the larger contents.

Adams and Leslie, who studied pH control in relation to the *B. p myxa* fermentation, advocated the use of ammonia because it elimin

sadvantages resulting from the use of calcium carbonate, because
bundant and relatively inexpensive, because it is usually free from
rganisms, and because it may be handled conveniently. They found
he maintenance of a pH range of 5.8 to 6.0 with ammonia was
um. It was favorable for the production of 2,3-butanediol and
ol; it minimized acid production and decreased the amount of
nia required. The use of ammonia did not affect adversely the
diol/ethanol ratio, which was about 1.5.

PERATURE. The temperatures most favorable for the production
butanediol and ethanol by *B. polymyxa* appear to lie close to 30°C.
gham, Adams, and Stanier suggested that a temperature of 32.5°C.
be most satisfactory, while Katznelson incubated his mashes at
A temperature of 30°C. has been used most frequently in the inves-
ns carried out by the Canadian National Research Laboratories.

ACE/VOLUME RATIO. The effect of the ratio of surface area to vol-
f fermentation mashes was first investigated by Katznelson, who
that the yields of 2,3-butanediol and ethyl alcohol were decreased
quantities of mash in 4-liter Erlenmeyer flasks were increased from
l. to 2,000 ml. by increments of 500 ml. The ratio of butanediol to
ol was also found to decrease as the volume of mash increased. The
t yields and shortest fermentation periods occurred when the flasks
ned shallow layers of mash.

ms and Leslie have confirmed the observation made by Katznel-
d made other significant contributions concerning the effects of the
e/volume ratio and reduced pressure on the fermentations by *B.*
*yxa* and *A. aerogenes*. As a result of many experiments, they found
he yields of 2,3-butanediol and ethanol were increased in media
were fermented in shallow layers, under both aerobic and anaerobic
ons. However, under anaerobic conditions (produced by the use
ogen gas), the yields of ethanol were greater and those of 2,3-
diol were smaller than those obtained from controls fermented in
sence of air.

results obtained by Adams and Leslie in determining the effect of
and anaerobic conditions are shown in Table 117. In carrying out
ts that led to the results tabulated, 300-ml. portions of wheat mash,
er cent concentration and containing 1 per cent of calcium car-
, were placed in 500-, 2,000- and 6,000-ml. Erlenmeyer flasks,
ed, inoculated with 3 per cent of inoculum (a 5 per cent whole-
-yeast-extract–calcium carbonate medium), and incubated at 30°C.
gen was passed over the surface of the mashes in one series of experi-
while the mashes of the control series were allowed to ferment in
esence of the gases evolved.

r experiments carried out by Adams and Leslie indicated that
under an atmosphere of carbon dioxide fermented at about the
ate regardless of the depth of the medium. The fact that fermen-
was inhibited under an atmosphere of carbon dioxide in mashes
large surface/volume ratio suggested that rapid fermentation of
in thin layers was due to the escape of carbon dioxide.

Table 117. Effect of Fermenting 300 ml. of Whole-wheat Mash with *polymyxa* under Aerobic and Anaerobic Conditions at Various Surface Ratios *

| Fermentation period, hr. | Surface cm./volume, ml. | Butanediol, per cent | | Ethanol, per cent | | Total products, per cent | | Rat B/ |
|---|---|---|---|---|---|---|---|---|
| | | Air | Nitrogen | Air | Nitrogen | Air | Nitrogen | Air |
| 24 | 0.16 | 0.78 | 0.93 | 0.41 | 0.53 | 1.19 | 1.46 | 1.90 |
| | 0.66 | 1.53 | 1.36 | 0.65 | 0.83 | 2.18 | 2.19 | 2.35 |
| | 1.32 | 1.96 | 1.62 | 0.76 | 1.07 | 2.72 | 2.69 | 2.58 |
| 48 | 0.16 | 1.96 | 1.62 | 0.84 | 1.26 | 2.80 | 2.88 | 2.33 |
| | 0.66 | 2.75 | 2.54 | 1.38 | 1.77 | 4.13 | 4.31 | 2.00 |
| | 1.32 | 2.93 | 2.66 | 1.38 | 1.76 | 4.26 | 4.42 | 2.20 |
| 72 | 0.16 | 2.22 | 2.03 | 1.17 | 1.26 | 3.39 | 3.29 | 1.90 |
| | 0.66 | 2.93 | 2.54 | 1.39 | 1.77 | 4.32 | 4.31 | 2.11 |
| | 1.32 | 3.06 | 2.66 | 1.22 | 1.76 | 4.28 | 4.42 | 3.06 |

* G. A. Adams and J. D. Leslie, *Can. Jour. Research,* **F, 24**: 107 (1946).

effects of reduced pressures. Experiments were also carried o Adams and Leslie to determine the effect of reduced pressure on th of fermentation and yield of products by *B. polymyxa* from 15 per whole-wheat mashes. Typical results are shown in Table 118. In ge

Table 118. Effect of Reduced Pressure on Rate of Fermentation and Pr Yield by *Bacillus polymyxa* *

| Fermentation time, hr. | Pressure, in. of Hg | Butanediol, per cent | Ethanol, per cent | Total products, per cent | Ratio | p |
|---|---|---|---|---|---|---|
| 24 | 30 | 0.85 | 0.51 | 1.36 | 1.67 | 6 |
| | 10 | 1.66 | 1.14 | 2.80 | 1.45 | 5 |
| 48 | 30 | 1.76 | 1.03 | 2.79 | 1.71 | 6 |
| | 10 | 2.44 | 1.92 | 4.36 | 1.27 | 5 |
| 72 | 30 | 2.47 | 1.52 | 3.99 | 1.62 | 5 |
| | 10 | 2.51 | 1.96 | 4.47 | 1.28 | 5 |
| 96 | 30 | 2.65 | 1.73 | 4.38 | 1.53 | 5 |
| | 10 | 2.52 | 2.02 | 4.45 | 1.25 | 5 |
| 120 | 30 | 2.76 | 1.78 | 4.54 | 1.55 | 5 |
| | 10 | 2.52 | 2.06 | 4.58 | 1.23 | 5 |

* G. A. Adams and J. D. Leslie, *Can. Jour. Research,* **F, 24**: 107 (1946).

the effect of the use of low pressures was to reduce the fermentation and the butanediol/ethanol ratios. Fermentations under reduced pre were about 95 per cent complete in 48 hr., whereas those at atmosp pressure were only about 60 per cent complete. Ethanol produ increased at the expense of 2,3-butanediol production; however, the yields of the two products under reduced pressure approached the ·tical yield of 4.6 per cent. It is noteworthy also that the pH ed to a low of 5.78 in 24 hr. and then increased until the end of the ntation, a fact that Adams and Leslie suggested might be due to the ›lytic breakdown of wheat proteins to yield ammonia.

OBIC AND ANAEROBIC FERMENTATIONS. The effect of passing various through the fermentation medium was studied by Adams (1946). ion was accomplished by passing 333 ml. of gas per minute per liter sh through the medium. The gases used were air, oxygen, nitrogen, gen, and carbon dioxide.

ler aerobic conditions, which were obtained by the passage of air or n through the media, the yields of 2,3-butanediol were increased, ose of ethanol were decreased in comparison with the controls which neither aerated nor oxygenated. Oxygenation and aeration increased ıtanediol/ethanol ratio and the rate of the formation of butanediol, did not shorten the fermentation period.

ler anaerobic conditions, which were produced by the passage of en or hydrogen gases through the media, the results were somewhat ·nt. Both nitrogen and hydrogen gases increased the rate of formand final yield of ethanol.

· results of the fermentation of 15 per cent whole-wheat mashes aerobic and anaerobic conditions are shown in Table 119. It may

119. FERMENTATION OF 15 PER CENT WHOLE-WHEAT MASH UNDER AEROBIC AND ANAEROBIC CONDITIONS BY *Bacillus polymyxa* *

| ·m | Aeration treatment | Butanediol, per cent | | | | | Ethanol, per cent | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Time, hr. | | | | | Time, hr. | | | | |
| | | 24 | 48 | 72 | 96 | 120 | 24 | 48 | 72 | 96 | 120 |
| ) | Control | 0.76 | 1.85 | 2.44 | 2.60 | 2.70 | 0.48 | 1.13 | 1.55 | 1.68 | 1.69 |
| | Air | 0.86 | 1.91 | 2.68 | 2.91 | 2.88 | 0.44 | 0.86 | 1.25 | 1.33 | 1.31 |
| | $O_2$ | 1.13 | 2.73 | 3.06 | 3.04 | 3.04 | 0.42 | 0.95 | 1.08 | 1.10 | 1.09 |
| | $N_2$ | 1.31 | 2.33 | 2.44 | 2.47 | 2.48 | 0.86 | 1.82 | 1.88 | 1.87 | 1.85 |
| | $CO_2$ | 1.65 | 1.93 | 2.40 | 2.52 | 2.59 | 0.45 | 1.23 | 1.73 | 1.90 | 1.81 |
| | $H_2$ | 0.93 | 1.99 | 2.43 | 2.59 | 2.62 | 0.65 | 1.71 | 1.86 | 1.91 | 1.91 |
| ) | Control | 0.67 | 1.47 | 2.18 | 2.51 | 2.71 | 0.40 | 0.81 | 1.23 | 1.51 | 1.65 |
| | Air | 0.61 | 1.36 | 2.28 | 2.90 | 3.09 | 0.30 | 0.55 | 0.86 | 1.05 | 0.98 |
| | $O_2$ | 1.02 | 2.03 | 2.88 | 2.95 | 3.05 | 0.36 | 0.56 | 0.80 | 0.87 | 0.94 |
| | $N_2$ | 1.10 | 2.47 | 2.51 | 2.49 | 2.51 | 0.73 | 1.76 | 1.88 | 1.85 | 1.83 |
| | $CO_2$ | 0.70 | 1.62 | 2.35 | 2.46 | 2.51 | 0.45 | 1.00 | 1.46 | 1.62 | 1.61 |
| | $H_2$ | 0.91 | 2.13 | 2.51 | 2.54 | 2.54 | 0.56 | 1.44 | 1.78 | 1.90 | 1.90 |

A. Adams, *Can. Jour. Research,* **F**, 24: 1 (1946).

erved from an examination of the table that the use of carbon dioxsulted in yields of butanediol and ethanol which were similar to obtained when nitrogen and hydrogen gases were used and that the ntations were largely completed in 72 hr.

EFFECT OF AGITATION. Adams investigated the effect of continu intermittent agitation by mechanical shakers on the product 2,3-butanediol and ethanol by *B. polymyxa*. He found that cont agitation inhibited the production of 2,3-butanediol and ethanol (p larly the ethanol) and increased the butanediol/ethanol ratio. I with intermittent agitation were similar.

EFFECT OF YEAST-EXTRACT AND WHEAT FRACTIONS. The addition o extract to a whole-wheat mash may stimulate the production butanediol, according to Katznelson. The largest degree of stimu was produced by 1 g. of yeast extract per 15 g. of whole wheat in a cent mash. The ashing of the yeast extract destroyed its stimu properties. Ledingham, Adams, and Stanier demonstrated (ref Table 120) that yeast extract in 0.25 per cent concentration increas

TABLE 120. EFFECT OF WHEAT FRACTIONS AND YEAST EXTRACT ON FERMENTATI

| Wheat fraction | 2,3-Butanediol + ethano per cent | | | |
|---|---|---|---|---|
| | Strain 233b (2) | | Strain 5 | |
| | Yeast extract added | | | |
| | None | 0.25 per cent | None | pe |
| Whole-wheat flour | 3.33 | 3.56 | 3.17 | 3 |
| Starch + wash water | 2.49 | 3.00 | 2.56 | 3 |
| Starch + wash water + bran | 2.90 | 3.26 | 3.00 | 3 |
| Whole-wheat flour | 2.78 | 3.29 | 3.12 | 3 |
| Starch + wash water + bran | 2.68 | 3.00 | 2.93 | 3 |
| Starch + wash water + bran + half the gluten | 2.71 | 2.90 | 2.82 | 3 |
| Starch + wash water + bran + all the gluten | 2.78 | 3.01 | 2.80 | 2 |

* G. A. Ledingham, G. A. Adams, and R. Y. Stanier, *Can. Jour. Research*, F 48 (1945).

yield of butanediol and ethanol from whole-wheat flour and other w fractions. However, it was found that a concentration of greater 1 per cent yeast extract could decrease the rate of fermentation. The of 3 per cent corn-steep liquor produced results similar to those obta with the use of 0.25 to 0.5 per cent yeast extract.

As the result of research to determine which constituents of w wheat were essential for the fermentation medium, it was ascertaine Ledingham and his associates that the bran, shorts, germ, and sol nitrogen constituents were essential for a normal fermentation. H ever, it was found that the removal of gluten had no particular effec the results.

EFFECT OF GROWTH FACTORS ON THE FERMENTATION OF STARCH. Alth wheat starch with inorganic supplements is only partially utilize *B. polymyxa*, Fratkin and Adams (1946) found that 8 per cent st es could be fermented in a satisfactory manner after the addition of ole organic nutrients. For example, a starch–whole-wheat–wash- mash supplemented with 1 per cent of malt sprouts or 2.5 per cent orts appeared to yield best results. Table 121 summarizes some of ndings of Fratkin and Adams with respect to the use of various

121. EFFECT OF VARIOUS SUPPLEMENTS IN VARYING CONCENTRATIONS IN A STARCH–WHOLE-WHEAT–WASH-WATER MASH [a] FERMENTATION [b]

| Nutrient | Per cent | 2,3-Butanediol + ethanol, per cent | | |
|---|---|---|---|---|
| | | 48 hr. | 72 hr. | 96 hr. |
| Control | | 1.61 | 2.99 | 3.52 |
| prouts:[c] | | | | |
| lized | 1.0 | 2.95 | 4.03 | 4.21 |
| erilized | 0.5 | 3.43 | 0.76 [d] | 0.80 [d] |
| [e] | 1.0 | 2.01 | 3.64 | 4.11 |
| | 1.0 | 2.19 | 3.57 | 4.16 |
| as [g] | 1.0 | 2.64 | 3.61 | 4.03 |
| | 1.0 | 2.49 | 3.87 | 4.06 |
| ns | 1.0 | 1.48 | 3.13 | 3.80 |
| extract | 0.5 | 2.72 | 3.75 | 3.89 |
| teep liquor | 0.5 | 2.09 | 3.13 | 3.54 |
| prouts: | | | | |
| lized | 2.0 | 3.14 | 4.25 | 4.45 |
| erilized | 1.0 | 3.61 | 4.24 | 4.11 [d] |
| | 2.0 | 1.95 | 3.80 | 4.23 |
| | 2.0 | 2.03 | 3.68 | 4.22 |
| s | 2.0 | 2.85 | 3.85 | 4.18 |
| | 2.0 | 2.94 | 3.77 | 4.01 |
| ns | 2.0 | 2.03 | 3.58 | 4.12 |
| xtract | 1.0 | 2.88 | 3.75 | 3.96 |
| eep liquor | 1.0 | 2.47 | 3.51 | 3.80 |
| routs: | | | | |
| ized | 3.0 | 3.44 | 4.36 | 4.48 |
| rilized | 1.5 | 3.37 | 2.40 [d] | 2.39 [d] |
| | 3.0 | 2.24 | 4.11 | 4.28 |
| | 3.0 | 2.05 | 3.54 | 4.22 |
| s | 3.0 | 2.90 | 3.87 | 4.17 |
| | 3.0 | 3.17 | 4.15 | 4.21 |
| ns | 3.0 | 1.99 | 3.64 | 4.06 |
| extract | 1.5 | 2.87 | 3.58 | 3.95 |
| eep liquor | 1.5 | 1.93 | 3.16 | 3.57 |

ch content of mash, 7.93 per cent.

. Fratkin and G. A. Adams, *Can. Jour. Research*, **F**, **24**: 29 (1946).

nentables in malt sprouts, 22.59 per cent.

taminated.

ch content of shorts, 11.32 per cent.

ch content of bran, 7.88 per cent.

gras—dehydrated young oat plants; obtained from Greenmelk Co., Ltd., burg, Ont.

nutrients, such as malt sprouts, shorts, bran, cerogras, alfalfa, s
yeast extract, and corn-steep liquor, in different concentration, in
whole-wheat–wash-water mash fermentations.

PRODUCTION FROM CORNSTARCH. Kooi, Fulmer, and Underkofler
reported on a procedure for the successful production of 2,3-but
from mashes containing cornstarch and nutrients by *B. polymyx*
best medium contained 7.5 g. of commercial cornstarch, 0.5 g.
gluten, 0.006 g. of potassium permanganate, and 0.5 g. of calcium
nate per 100 ml. The yields from this medium (26.8 per cent 2,3-
diol, 1.1 per cent acetoin, and 14.8 per cent ethanol) were compar
those obtained from corn mashes of an equivalent starch content.

It was found that increased yields of 2,3-butanediol were obtaine
cornstarch and corn mashes when 0.5 per cent of dried brewers' y
malt sprouts was added to the corn-mash inoculum medium.

EFFECT OF GROWTH FACTORS AND NITROGEN SOURCES ON THE FER
TION OF A GLUCOSE–INORGANIC-SALT MEDIUM. The nutritional r
ments of *B. polymyxa* have been studied by Katznelson and Lo
(1944). The basal inorganic medium used by them contained 1.
$K_2HPO_4$, 1.0 g. of $KH_2PO_4$, 0.2 g. of $MgSO_4 \cdot 7H_2O$, 0.1 g. of NaCl,
of $CaCl_2$, 0.01 g. of $FeSO_4 \cdot 7H_2O$, 0.01 g. of $MnSO_4 \cdot 4H_2O$, and 0.0
$ZnSO_4$ in 1,000 ml. of distilled water. The salt solution was filtere
being heated to boiling and 5.0 g. of c.p. glucose and 2 g. of vitami
casein hydrolyzate were then added. The pH was adjusted to 6.8
resultant medium was distributed into thoroughly cleaned and
culture tubes and sterilized for 15 min. at a steam pressure of
per sq. in.

Studies concerned with growth factor requirements revealed th
strains of *B. polymyxa* would grow in the medium described above
when biotin was present, which established the essentiality of this vit
The growth of some strains was stimulated by thiamin, whereas t
others was inhibited. Inositol, nicotinic acid, pantothenic acid, py
ine, and riboflavin appeared to be without effect on the growth of s
of *B. polymyxa*. Yeast extract, in a concentration of 3 g. per liter
duced better growth in the medium containing only the inorganic salt
glucose than all the other growth factors examined.

The nitrogen requirements of strains of *B. polymyxa* were also in
gated by Katznelson and Lochhead. Although hydrolyzed casein
be replaced by ammonium sulfate, urea, and a mixture of 18 amino
these substances were in general inferior to casein hydrolyzate. As
gine and potassium nitrate were found to be poor sources of nitr
Peptone and tryptone were about as effective as the casein hydroly
sources of nitrogen. Yeast extract produced the most abundant gr
part of which may be attributed to growth factors it contained.

EFFECT OF BACTERIOPHAGE. The effect of bacteriophage on stra
*B. polymyxa*, particularly in relation to the production of 2,3-buta
has been studied by Katznelson. Freshly isolated bacteriophages a
old mixed bacteriophage, which could be separated into eight di
groups based on differences in size and internal structure of the pl

used in the investigation, as well as 82 strains of *B. polymyxa* (44 ›m freshly isolated).

en the 82 strains of *B. polymyxa* were tested with a mixture of all ifferent types of bacteriophage, complete lysis of 63 strains and ıl lysis of 6 strains took place. There appeared to be no relationship en the ability to produce 2,3-butanediol and susceptibility to bac- hage in the cases of the strains of *B. polymyxa* examined.

› effect of temperature on bacteriophage was studied by Katznelson, ound that a temperature of 55°C. for 30 min. inactivated it. How- it was ascertained that bacteriophage may be transmitted through rial spores and in one case a temperature of 80°C. for 60 min. failed troy it. Temperatures sufficiently high to destroy the bacteriophage estroyed the spores of the strain of *B. polymyxa* tested.

order to avoid the serious problems associated with bacteriophage night arise in a production plant, Katznelson advocated the ade- sterilization of equipment and materials, the employment of aseptic .ques, and the use of cultures shown by careful examination to be f bacteriophage and nonlysogenic. He also suggested that strains of *'ymyxa* that formed spores be used and that the inoculum be pas- ed. As a further precautionary measure, he suggested the use of ›culum containing a combination of three resistant strains of *B. poly-* when bacteriophage was known to be present. Although mixtures › or three highly resistant strains have produced good results, a single ›tible strain has usually produced better yields of 2,3-butanediol. ombination of five or more strains (for example, of two susceptible ıree resistant strains) has not produced a good inoculum.

ʼT-PLANT PRODUCTION UNIT. A small-scale process for producing tanediol has been described by Rose and King (1945). Their pro- n unit has a capacity for about 25 gal. of whole-wheat mash per

HEMISTRY OF THE FERMENTATION. Studies concerning the biochem- ıf the fermentation brought about by strains of *B. polymyxa* have ıade by Donker in 1926, by Stahly and Werkman in 1942, and by ; and Stanier in 1945.

-ıly and Werkman (1942) grew *B. polymyxa* in a medium containing ent glucose, 0.5 per cent peptone, 0.2 per cent dipotassium hydrogen ıate, and 1 per cent calcium carbonate at a temperature of 30°C. e fixation of aldehyde, 1 per cent calcium sulfite ($CaSO_3$) was used. · and Werkman observed that the addition of acetaldehyde to the e medium resulted in larger yields of ethanol, acetylmethylcarbinol ›in), and 2,3-butanediol and that the addition of acetic acid resulted ger yields of acetoin and 2,3-butanediol. They were therefore of the n that acetaldehyde was probably an intermediate in the fermenta- They also found that a low redox potential favored the production of tanediol whereas a high redox potential favored the production .oin.

ms and Stanier have prepared carbon balances for the anaerobic ıtations of glucose, xylose, pyruvic acid, and mannitol, using *B.*

*polymyxa*, strain NRC 25. Table 122 summarizes some of the inf
tion obtained by them concerning these fermentations.

Study of Table 122 discloses interesting facts regarding the re
weights and ratios of the products formed by the fermentations o

TABLE 122. FERMENTATION OF GLUCOSE, XYLOSE, PYRUVIC ACID, AND MA
BY *Bacillus polymyxa*, STRAIN NRC 25 *

| Raw material | Weights, g. | | | |
|---|---|---|---|---|
| | Glucose | Xylose | Pyruvic acid | Ma |
| Amount raw material fermented.. | 5.210 | 4.202 | 1.278 | 2. |
| Amounts of end products formed: | | | | |
| 2,3-Butanediol | 1.700 | 0.956 | None | 0. |
| Acetoin | 0.072 | 0.062 | 0.255 | 0. |
| Ethanol | 0.881 | 0.811 | Trace | 0.5 |
| Acetic acid | 0.050 | 0.129 | 0.455 | 0. |
| Lactic acid | ...... | ...... | Trace | 0.5 |
| Succinic acid | ...... | ...... | ...... | 0. |
| Carbon dioxide | 2.542 | 1.987 | 0.605 | 0.7 |
| Hydrogen | 0.041 | 0.046 | 0.016 | 0. |
| Carbon recovery, per cent | 101.6 | 92.9 | 93.0 | 106.8 |
| $CO_2$ calculated/$CO_2$ observed | 1.026 | 0.938 | 0.974 | 0.9 |
| $H_2$ calculated/$H_2$ observed | 1.079 | 0.712 | 0.950 | 0.9 |
| O/R index | 0.989 | 0.960 | 0.971 | 0.9 |

* G. A. Adams and R. Y. Stanier, *Can. Jour. Research*, **B**, 23: 1 (1945).

respective raw materials—glucose, xylose, pyruvic acid, and manni
The 2,3-butanediol/ethanol ratio may be computed by comparing
moles of butanediol produced with those of ethanol. Since the molecu
weight of 2,3-butanediol is 90 and that of ethanol is 46, there would
90 g. of the former and 46 g. of the latter produced when the ratio is 1

The authors suggested that pyruvic acid was dissimilated anaerobica
by *B. polymyxa* according to the following scheme:

$$\underset{\text{Pyruvic acid}}{CH_3 \cdot CO \cdot COOH} + H_2O \rightarrow \underset{\text{Acetic acid}}{CH_3 \cdot COOH} + CO_2 + H_2$$

$$\underset{\text{Pyruvic acid}}{2CH_3 \cdot CO \cdot COOH} \rightarrow \underset{\text{Acetaldehyde}}{2CH_3 \cdot CHO} + 2CO_2$$

$$\underset{\text{Acetaldehyde}}{2CH_3 \cdot CHO} \rightarrow \underset{\text{Acetoin}}{CH_3 \cdot CO \cdot CHOH \cdot CH_3}$$

PRODUCTION OF ACETONE BY STRAINS OF *B. polymyxa*. Rose (1926) c
firmed the evidence presented by Donker that acetone is produced fr
dextrose in small amounts by certain strains of *B. polymyxa* un
anaerobic conditions. Table 123 from the report by Rose supplies d
concerning the products and amounts of each formed by the fermentati
of dextrose by two different strains of *B. polymyxa*.

BLE 123. FERMENTATION PRODUCTS OF TWO STRAINS OF *Bacillus polymyxa* *, †

| Product | C 2(3) | C 25 |
|---|---|---|
| Butanediol | 45.5 | 40.6 |
| Acetoin | 2.8 | 0.2 |
| Ethanol | 76.7 | 83.2 |
| Formic acid | 1.2 | 2.4 |
| Acetic acid | 14.0 | 6.3 |
| Lactic acid | 3.2 | 2.9 |
| Succinic acid | 1.8 | 1.6 |
| Malic acid | 0.5 | 0.2 |
| Carbon dioxide | 188.3 | 200.7 |
| Hydrogen | 90.1 | 97.9 |
| Acetone | Tr.(?) | 9.5 |
| Dextrose fermented | 5077 | 5066 |
| Carbon recovery, per cent | 97.2 | 98.3 |
| $CO_2$, calculated/observed | 1.013 | 1.00 |
| $H_2$, calculated/observed | 0.998 | 1.001 |
| O/R index | 0.987 | 1.001 |
| $\frac{\text{Acetoin + diol}}{\text{Ethanol}}$ (by weight) | 1.23 | 0.96 |

Rose, *Can. Jour. Research,* **F, 24**: 320 (1946).
lds expressed as millimoles per 100 mM. of dextrose fermented.

*he* A. hydrophila *Fermentation.* This fermentation is character-
y the production of the levorotatory and meso forms of 2,3-bu-
ol, ethanol, and lactic acid from sugars. It was investigated
anier and Adams (1944), of the Canadian National Research
il.

ORGANISM. The fermentation described herein is brought about
*hydrophila*, a Gram-negative, nonsporeforming, rod-shaped organ-
the family Pseudomonadaceae.

L MEDIUM. The basal medium used by Stanier and Adams con-
the following ingredients: 1.25 g. of yeast extract, 1.0 g. of calcium
ate, 0.025 g. of magnesium sulfate, 0.65 ml. of 1.0 *M* phosphate
(pH 7.4), and 250 ml. of tap water. The carbohydrates investi-
were sterilized separately in concentrated aqueous solutions and
dded to flasks containing the above basal medium (after their
ation and at the time of inoculation). The concentration of carbo-
tes used was 1 to 2 per cent.

IENTATION CONDITIONS. Nitrogen gas (free from oxygen) was
through each flask at a slow rate. The media were inoculated at
for 3 to 4 days.

UCTS OF FERMENTATION. The types of products formed and the
ies of each may be ascertained by reference to Table 124, which
rizes some data obtained by Stanier and Adams in determining
bon balances for the fermentation of glucose, xylose, and pyruvic
*A. hydrophila.*

Table 124. Fermentation of Glucose, Xylose, and Pyruvic Acid by *Aeron hydrophila* *

| | Glucose | | Xylose | | Pyruvic a | |
|---|---|---|---|---|---|---|
| | Weight, g. | No. of moles/ 100 moles of glucose fermented | Weight, g. | No. of moles/ 100 moles of xylose fermented | Weight, g. | N mo 1 mo pyr aci me |
| Amount of raw material fermented | 5.490 | .... | 4.010 | .... | 3.530 | |
| Amount of end product formed: | | | | | | |
| 2,3-Butanediol | 1.502 | 54.7 | 0.939 | 39.0 | 0.125 | 3 |
| Acetoin | 0.047 | 1.7 | 0.061 | 2.6 | 0.021 | 0. |
| Ethanol | 0.730 | 52.0 | 0.600 | 48.9 | 0.038 | 2 |
| Acetic acid | 0.085 | 4.6 | 0.150 | 9.3 | 1.800 | 74. |
| Lactic acid | 0.639 | 23.3 | 0.491 | 20.4 | 0.776 | 21. |
| Succinic acid | 0.130 | 3.6 | 0.036 | 1.1 | | |
| $CO_2$ | 2.230 | 166.2 | 1.584 | 134.7 | 1.479 | 83. |
| $H_2$ | 0.035 | 57.5 | 0.029 | 53.9 | 0.035 | 43. |
| Carbon recovery, per cent | 98.2 | .... | 96.6 | .... | 106.3 | |
| $CO_2$ calculated/$CO_2$ observed | 0.997 | .... | 1.041 | .... | 1.013 | |
| $H_2$ calculated/$H_2$ observed | 1.108 | .... | 1.126 | .... | 1.102 | |
| O/R index | 1.021 | .... | 0.988 | .... | 1.052 | |
| 2,3-Butanediol/ethanol (approx.) | 1/1 | .... | 0.8/1 | .... | 1.6/1 | |

* R. Y. Stanier and G. A. Adams, *Biochem. Jour.*, **38**: 168 (1944).

FERMENTATION OF PYRUVIC ACID. Stanier and Adams suggested t pyruvic acid was fermented by *A. hydrophila* in accordance with the th following reactions:

$$\underset{\text{Pyruvic acid}}{3CH_3\cdot CO\cdot COOH} + 3H_2O \rightarrow \underset{\text{Acetic acid}}{3CH_3\cdot COOH} + 3CO_2 + 6H$$

$$CH_3\cdot CO\cdot COOH + 2H \rightarrow \underset{\text{Lactic acid}}{CH_3\cdot CHOH\cdot COOH}$$

$$4H \rightarrow 2H_2$$

4. *The* B. subtilis *Fermentation.* *B. subtilis* (Ford's strain) produ 2,3-butanediol and glycerol as the principal end products when grown a medium containing 3 per cent glucose, 1 per cent yeast extract, a 1 per cent calicum carbonate when the pH is maintained within the ran

) to 6.8, when the temperature is 30°C., and when the conditions are
robic, according to Neish, Blackwood, and Ledingham (1945). The
utanediol appears to be a mixture of the levorotatory and meso
in about equal quantities.

e fermentation is quite complex in that the course of the fermenta-
nay be sharply altered by what appear to be rather small changes in
onditions of fermentation, as, for example, changes in the thiamin
nt of the medium, the pH, oxygen relationships, or perhaps in the
is of the organism used (Neish et al., 1945).

TORICAL. Desmots in 1904 reported that *B. subtilis* formed acetoin
cultured on media rich in carbohydrates. Lemoigne in 1912 showed
*B. subtilis* produced 2,3-butanediol in the fermentation of carbo-
ite-rich media and that acetoin was produced from the former by
rial oxidation. Friedemann found that lactic acid was the chief
ct resulting from the fermentation of a medium composed of glucose,
extract, and peptone, having an initial pH of 7.6 (buffered with
hate). Acetic acid, formic acid, and ethanol were also formed.
alis (1944) has shown that thiamin exerts a very strong influence on
urse of the fermentation. For example, he found that 70 to 90 per
f the glucose of a medium was converted to lactic acid when the
in content was very small or absent. When thiamin was added to
utrient glucose medium, 2,3-butanediol was formed in significant
ities (26 moles per 100 moles of glucose) and the yield of lactic acid
ed to 50 per cent based on the glucose. The foregoing facts will
ome indication of the complexity of the fermentation. Neish, Black-
and Ledingham studied extensively (1945*a*, *b*) the dissimilation
cose by *B. subtilis* (Ford's strain).

ORGANISM. Neish, Blackwood, and Ledingham (1945*b*) used in
studies the Ford strain of *B. subtilis* (N.C.T.C. 2586), which is
d in the American Type Culture Collection as No. 9789.

INOCULUM. *B. subtilis* was grown in nutrient broth at 30°C. for
prior to use. Fermentation media were inoculated with 3 per cent
ulum.

CTS OF CONDITIONS OF FERMENTATION. Fermentations were carried
Neish, Blackwood, and Ledingham under a variety of conditions
th different results.

edium containing 3 per cent glucose, 1 per cent yeast extract, and
ent calcium carbonate at a temperature of 30°C., and at a pH of 6.8
, appeared to be especially favorable for the production of 2,3-
diol and glycerol when 300-ml. amounts in 1,000-ml. Erlenmeyer
were aerated with nitrogen gas at the rate of 100 ml. per min.
these conditions, 56.36 mM. of 2,3-butanediol, 39.91 mM. of glyc-
9.96 mM. of lactic acid, 12.88 mM. of ethanol, and 5.56 mM. of
acid were produced per 100 mM. of glucose dissimilated. Under
ne conditions (except that the medium was aerated with bubbles of
gas instead of nitrogen gas) 33.35 mM. of 2,3-butanediol, 33.65
f acetoin, 7.38 mM. of ethanol, 4.99 mM. of acetic acid, 3.66 mM.

of glycerol, and small quantities of lactic, formic, and *n*-butyric ac produced per 100 mM. of glucose dissimilated. Carbon dioxide, molecular hydrogen, was produced from the glucose in each case.

The effect of pH on the dissimilation of glucose is shown in Tab It will be noticed that at the higher pH range (7.6 to 6.8) ther

TABLE 125. EFFECT OF pH ON THE DISSIMILATION OF GLUCOSE *, †, ‡

| Product | mM./100 mM. of glucose fermented | |
|---|---|---|
| | Grown at pH 6.2 to 5.8 | Grown at pH 7.6 to 6.8 |
| 2,3-Butanediol | 56.15 | 36.16 |
| Acetoin | Trace | Trace |
| Glycerol | 26.28 | 16.39 |
| Ethanol | 18.24 | 28.70 |
| Lactic acid | 39.13 | 53.08 |
| Succinic acid | Trace | 5.05 |
| Formic acid | 9.97 | 30.14 |
| Acetic acid | Doubtful | 3.98 |
| *n*-Butyric acid | Doubtful | 2.76 |
| Carbon dioxide (calculated) | 130.56 | 101.01 |
| Carbon accounted for | 100.0 per cent | 97.0 per cent |
| Glucose dissimilated (4 days) | 73.7 per cent | 68.2 per cent |

* A. C. Neish, A. C. Blackwood, and G. A. Ledingham, *Can. Jour. Research,* 290 (1945).

† Medium: Glucose, 3 per cent; potassium dihydrogen phosphate, 0.5 per dipotassium hydrogen phosphate, 0.6 per cent; magnesium sulfate heptahydrat per cent; casein hydrolyzate, 0.1 per cent.

‡ Conditions: Grown at 30°C. without aeration; the pH was measured with a electrode every 8 to 10 hr. and adjusted with *N* sodium hydroxide.

decreases in the quantities produced of 2,3-butanediol and glycerol, increases in the quantities of ethanol, lactic acid, succinic acid, fo acid, acetic acid, and *n*-butyric acid in comparison with the amo produced of these same items at the lower pH range (6.2 to 5.8).

In general, a high pH favored the production of acids and ethan the expense of the 2,3-butanediol and glycerol, while a low pH fav the production of 2,3-butanediol and glycerol.

A medium containing 1 per cent glucose, 1.1 per cent peptone, 0.3 cent meat extract, and 1.5 per cent disodium hydrogen phosphate (Fri mann's medium) was fermented without aeration at a temperatur 30°C. and with an initial pH of 7.6 and a final pH of 6 and result the production of 98.50 mM. of lactic acid and 20.43 mM. of butanediol per 100 mM. of glucose dissimilated, according to Neish, Bl wood, and Ledingham.

5. *The* S. marcescens *Fermentation.* This fermentation was studied by Pederson and Breed (1928). They used a medium that

d 1 per cent peptone broth and 3 per cent glucose, and which was red with an excess of sterile calcium carbonate. Using culture 1377 of *S. marcescens,* they obtained the following percentages of end ucts, based on the sugar fermented: 39.8 per cent lactic acid, 28 per carbon dioxide, 17.7 per cent acetoin and 2,3-butanediol, 5.5 per cent c acid, 4.7 per cent ethanol, 1.3 per cent succinic acid, 0.2 per cent ic acid, and a small amount of hydrogen gas. Similar end products produced by other strains of *S. marcescens* and *S. indica.*

ish and his associates (1947) investigated fermentations carried out r anaerobic conditions, using four strains of *S. marcescens* that pro- l little or no hydrogen. They used a yeast extract medium (150 ml. 1-liter Erlenmeyer flask) that contained 5 per cent glucose, 0.5 per yeast extract, 0.05 per cent potassium dihydrogen phosphate, 0.05 per potassium monohydrogen phosphate, and 0.02 per cent magnesium te. This medium was buffered with 2 per cent calcium carbonate, ately sterilized. Anaerobic conditions were produced by continu- passing oxygen-free nitrogen through the medium. The fermenta- were carried out at 35°C. Results obtained are shown in Table 126.

TABLE 126. ANAEROBIC DISSIMILATION OF GLUCOSE BY *Serratia marcescens**

| Products | mM. of products/100 mM. of glucose dissimilated | | | |
|---|---|---|---|---|
| | Strain S 1 | Strain S 2 | Strain S 3 | Strain S 9 |
| tanediol | 57.90 | 55.20 | 51.45 | 42.45 |
| n | 0.25 | 0.50 | 0.81 | 1.14 |
| ol | 6.14 | 4.18 | 4.54 | 5.63 |
| l | 40.85 | 41.30 | 42.24 | 25.90 |
| acid | 15.70 | 26.50 | 33.09 | 54.15 |
| acid | 48.50 | 44.00 | 39.80 | 27.60 |
| acid | Nil | Nil | Nil | Nil |
| c acid | 2.98 | 3.34 | 3.41 | 18.80 |
| dioxide | 103.8 | 102.5 | 106.1 | 78.2 |
| en | Nil | Nil | 0.52 | 0.27 |
| tation time, days | 17 | 12 | 9 | 7 |
| dissimilated, per cent | 93.2 | 88.5 | 99.5 | 99.9 |
| accounted for, per cent | 91.4 | 93.0 | 94.6 | 97.5 |
| dex | 0.99 | 1.03 | 1.04 | 1.01 |

C. Neish, A. C. Blackwood, F. M. Robertson, and G. A. Ledingham, *Can. Jour.* *ch,* **B, 25**: 65 (1947).

**rification of Mashes.** Strohmaier and Lovell (1946) investigated larification of acid-hydrolyzed corn mashes and the fermented s of the same type (glycol beer) by filtration and centrifugation ds. They found that pH and temperature were very important s in connection with clarification. The optimum pH depended upon ture of the mash and was 5.0 and 6.2 with cooked (continuous s), unfermented mash and 4.8 for glycol beer produced on a pilot-plant scale. The rates of filtration were highest, at a constant pH, a highest temperature (210°F.).

The clarification of corn mashes by both filtration and centrifug could be facilitated by adding small amounts of a swelling type of tonite to the mashes at a pH of 2.0 to 3.0 and at high temperat according to the above investigators.

**Recovery of 2,3-Butanediol.** It is not a simple matter to rec 2,3-butanediol from the fermentation liquor (beer), on account of its boiling point (about 180°C.), its suspended and soluble solids, and presence of nonvolatile substances.

Blom, Reed, Efron, and Mustakas (1945) described a process for re ering 2,3-butanediol from fermentation liquors. In their process, the is concentrated to a sirup in evaporators and then steam-stripped a elevated pressure in a special column packed with ceramic balls.

The recovery of 2,3-butanediol from glycol beer as formal has b described by Senkus (1946). A flow sheet for glycol recovery by method is shown in Fig. 69.

In the laboratory, the fermented medium may be saturated with pot sium carbonate and then extracted with *n*-butanol, or continuou extracted with diethyl ether by the method of Kolfenbach, Kooi, Fuln and Underkofler (1944).

Other methods for recovering 2,3-butanediol have been reported Liebmann (1945); Othmer, Bergen, Schlechter, and Bruins (1945); R and King; and others.

**Determination of 2,3-Butanediol.** The quantitative determination the amount of 2,3-butanediol in a sample may be accomplished by periodate oxidation method, which was developed by Johnson, of Department of Biochemistry at the University of Wisconsin. The 2 butanediol is oxidized by periodic acid to form 2 molecules of acetal hyde. The aldehyde is fixed or absorbed by bisulfite. The result aldehyde-bisulfite addition product is treated with alkali and the bis fite determined by titration with a dilute iodine solution.

**Uses.** The uses of various forms of 2,3-butanediol have been d cussed by Liebmann (1945) and reviewed generally by Underkofler a Fulmer (1948). Reference has already been made to its potential use a source of synthetic rubber. In this process, the 2,3-butanediol may converted to 1,3-butadiene by a suitable method, such as by the pyroly of the diacetate. The levorotatory form [D(−)-2,3-butanediol] is a s isfactory antifreeze agent, according to Clendenning (1946). He a Wright (1946) reported that a mixture of 20 per cent of methanol, 40 p cent of butanediol, and 40 per cent of water was suitable for an antifre at temperatures as low as −50°C. Neish and MacDonald (1947) scribed the preparation and physical properties of cyclic acetals a ketals that were readily derived from levorotatory 2,3-butanediol. T 2,3-butanediols (and their derivatives) have potential uses as solven moisteners, and softeners, as has been mentioned previously.

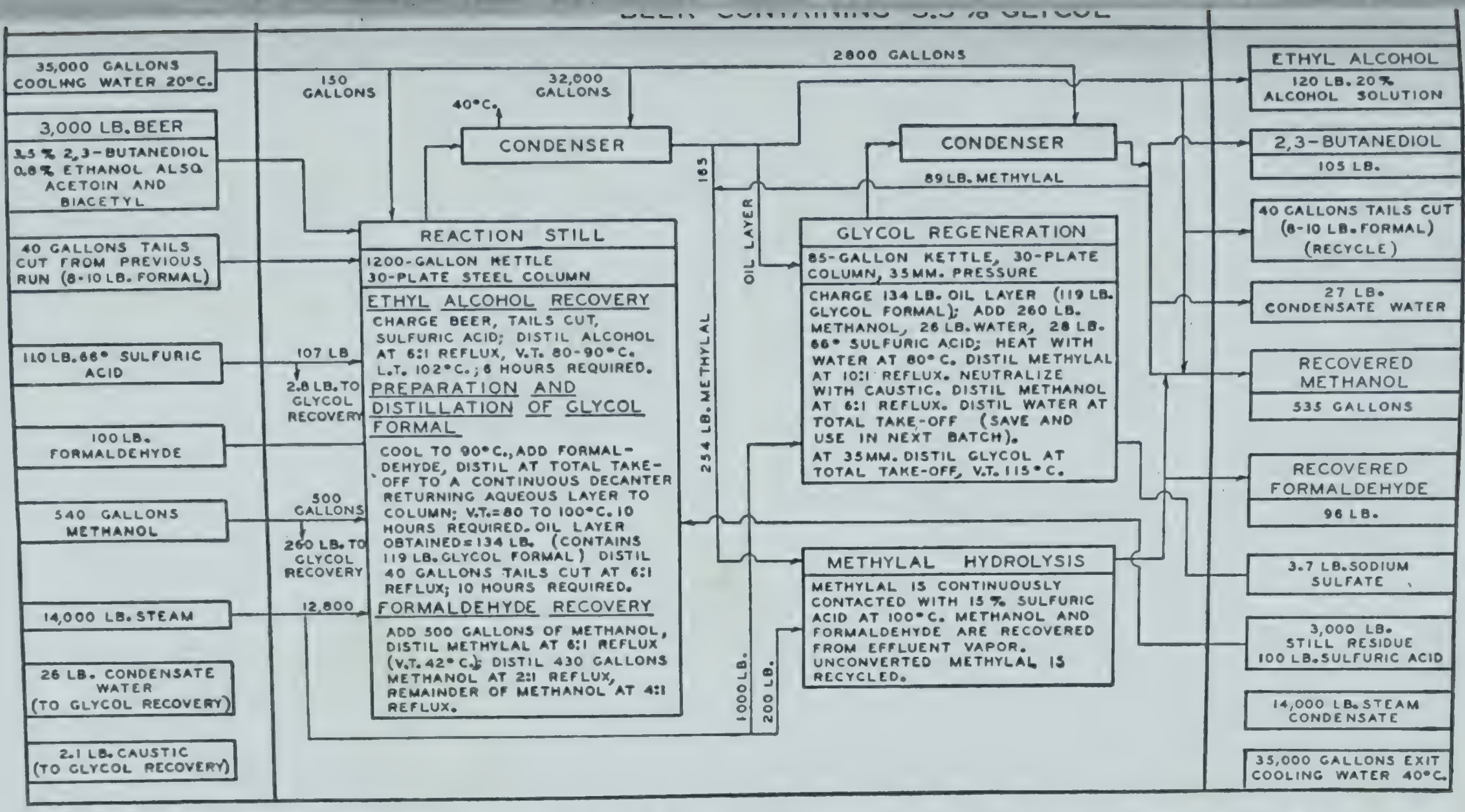

BEER CONTAINING 3.5% GLYCOL
35,000 GALLONS COOLING WATER 20°C.
3,000 LB. BEER
3.5% 2,3-BUTANEDIOL 0.8% ETHANOL ALSO ACETOIN AND BIACETYL
40 GALLONS TAILS CUT FROM PREVIOUS RUN (8-10 LB. FORMAL)
110 LB. 66° SULFURIC ACID
100 LB. FORMALDEHYDE
540 GALLONS METHANOL
14,000 LB. STEAM
26 LB. CONDENSATE WATER (TO GLYCOL RECOVERY)
2.1 LB. CAUSTIC (TO GLYCOL RECOVERY)
150 GALLONS
32,000 GALLONS
40°C.
2800 GALLONS
CONDENSER
107 LB
2.8 LB. TO GLYCOL RECOVERY
500 GALLONS
260 LB. TO GLYCOL RECOVERY
12,800
REACTION STILL
1200-GALLON KETTLE 30-PLATE STEEL COLUMN
ETHYL ALCOHOL RECOVERY
CHARGE BEER, TAILS CUT, SULFURIC ACID; DISTIL ALCOHOL AT 6:1 REFLUX, V.T. 80-90°C. L.T. 102°C.; 6 HOURS REQUIRED.
PREPARATION AND DISTILLATION OF GLYCOL FORMAL
COOL TO 90°C., ADD FORMALDEHYDE, DISTIL AT TOTAL TAKE-OFF TO A CONTINUOUS DECANTER RETURNING AQUEOUS LAYER TO COLUMN; V.T.=80 TO 100°C. 10 HOURS REQUIRED. OIL LAYER OBTAINED=134 LB. (CONTAINS 119 LB. GLYCOL FORMAL) DISTIL 40 GALLONS TAILS CUT AT 6:1 REFLUX; 10 HOURS REQUIRED.
FORMALDEHYDE RECOVERY
ADD 500 GALLONS OF METHANOL, DISTIL METHYLAL AT 6:1 REFLUX (V.T. 42°C.); DISTIL 430 GALLONS METHANOL AT 2:1 REFLUX, REMAINDER OF METHANOL AT 4:1 REFLUX.
165
254 LB. METHYLAL
OIL LAYER
1000 LB.
200 LB.
CONDENSER
89 LB. METHYLAL
GLYCOL REGENERATION
85-GALLON KETTLE, 30-PLATE COLUMN, 35 MM. PRESSURE
CHARGE 134 LB. OIL LAYER (119 LB. GLYCOL FORMAL); ADD 260 LB. METHANOL, 26 LB. WATER, 28 LB. 66° SULFURIC ACID; HEAT WITH WATER AT 80°C. DISTIL METHYLAL AT 10:1 REFLUX. NEUTRALIZE WITH CAUSTIC. DISTIL METHANOL AT 6:1 REFLUX. DISTIL WATER AT TOTAL TAKE-OFF (SAVE AND USE IN NEXT BATCH).
AT 35 MM. DISTIL GLYCOL AT TOTAL TAKE-OFF, V.T. 115°C.
METHYLAL HYDROLYSIS
METHYLAL IS CONTINUOUSLY CONTACTED WITH 15% SULFURIC ACID AT 100°C. METHANOL AND FORMALDEHYDE ARE RECOVERED FROM EFFLUENT VAPOR. UNCONVERTED METHYLAL IS RECYCLED.
ETHYL ALCOHOL
120 LB. 20% ALCOHOL SOLUTION
2,3-BUTANEDIOL
105 LB.
40 GALLONS TAILS CUT (8-10 LB. FORMAL) (RECYCLE)
27 LB. CONDENSATE WATER
RECOVERED METHANOL
535 GALLONS
RECOVERED FORMALDEHYDE
96 LB.
3.7 LB. SODIUM SULFATE
3,000 LB. STILL RESIDUE 100 LB. SULFURIC ACID
14,000 LB. STEAM CONDENSATE
35,000 GALLONS EXIT COOLING WATER 40°C.

FIG. 69. Flow sheet for glycol recovery. [*Courtesy of M. Senkus, Ind. Eng. Chem.*, 38: 913 (1946). *Copyright* 1946 *by the American Chemical Society and reprinted by permission of the copyright owner.*]

### References on the Production and Properties of 2,3-Butanediol

Adams, G. A.: Production and Properties of 2,3-Butanediol. VII. Fermenta Wheat by *Aerobacillus polymyxa* under Aerobic and Anaerobic Conditior *Jour. Research,* **F, 24**: 1–11 (1946).

——— and J. D Leslie: Production and Properties of 2,3-Butanediol. VIII. p trol in *Aerobacillus polymyxa* Fermentation and Its Effects on Produ Their Recovery, *Can. Jour. Research,* **F, 24**: 12–28 (1946).

——— and ———: Production and Properties of 2,3-Butanediol. X. The Eff Surface-Volume Ratio and Reduced Pressure on the Fermentation of hydrates by *Aerobacillus polymyxa* and *Aerobacter aerogenes, Can. Jo search,* **F, 24**: 107–118 (1946).

——— and R. Y. Stanier: Production and Properties of 2,3-Butanediol. III. on the Biochemistry of Carbohydrate Fermentation by *Aerobacillus pol* *Can. Jour. Research,* **F, 23**: 1–9 (1945).

Blackwood, A. C., and G. A. Ledingham: Production and Properties of 2,3-B diol. XX. Influence of the Inoculum on the *Aerobacillus polymyxa* Ferm tion, *Can. Jour. Research,* **F, 25**: 180–191 (1947).

Blom, R. H., D. L. Reed, A. Efron, and G. C. Mustakas: Recovery of 2,3-Bu Glycol from Fermentation Liquors, *Ind. Eng. Chem.,* **37**: 865–870 (1945).

Böeseken, J., and R. Cohen: La Configuration des Butanediols 2.3, *Rec. trav.* **47**: 839–848 (1928).

Breden, C. R.: The Chemical Action of *Aerobacter faeni* on Xylose and on Su doctoral thesis, Iowa State College, Ames, Iowa, 1930.

——— and E. I. Fulmer: *Iowa State Coll. Jour. Sci.,* **5**: 133–153 (1931).

Chappell, C. H.: A Study of 2,3-Butylene Glycol and Its Derivatives, thesis, State College, Ames, Iowa, 1935.

Christensen, L. M.: Increased Yield of 2,3-Butylene Glycol, U.S. Patent 2,34 Mar. 14, 1944.

Clendenning, K. A.: Production and Properties of 2,3-Butanediol. XI. Evaluati *Levo-* 2,3-Butanediol as a Non-Volatile Antifreeze Compound, *Can. Jour. search,* **F, 24**: 249–271 (1946).

———: Production and Properties of 2,3-Butanediol. XVI. Density, Optical Rot Power, and Refraction of Aqueous 2,3-Butanediol Solutions, *Can. Jour. Rese* **B, 24**: 269–279 (1946).

——— and D. E. Wright: Production and Properties of 2,3-Butanediol. XII. freeze Properties of Ternary Aqueous Solutions Containing *Levo-* 2,3-Butan as a Major Component, *Can. Jour. Research,* **F, 24**: 287–299 (1946).

Donker, H. J. L.: "Bijdrage tot de kennis der boterzuurbutylalcohol en aceto tingen," thesis, Delft, 1926.

Elder, A. L.: Progress of Butadiene Production, *Ind. Eng. Chem.,* **34**: 1260- (1942).

Fratkin, S. B., and G. A. Adams: Production and Properties of 2,3-Butane IX. The Effect of Various Nutrient Materials on the Fermentation of Starc *Aerobacillus polymyxa, Can. Jour. Research,* **F, 24**: 29–38 (1946).

Fulmer, E. I.: The Physico-Chemical Approach to Problems of Fermenta *Brewers Dig.,* **18**(8): 29–32; **18**(9): 37–39, 44 (1943).

———, A. C. Bantz, and L. A. Underkofler: The Use of Alfalfa Extract to Su Nutrients for the Growth and Chemical Activities of *Aerobacter suboxy* *Iowa State Coll. Jour. Sci.,* **18**: 369–376 (1944).

———, L. M. Christensen, and A. R. Kendall: Production of 2,3-Butylene Glyc Fermentation. Effect of Sucrose Concentration, *Ind. Eng. Chem.,* **25**: 798 (1933).

———, L. A. Underkofler, and A. C. Bantz: The Production of Acetylmethylcarb by the Action of *Acetobacter suboxydans* upon 2,3-Butylene Glycol, *Jour. Chem. Soc.,* **65**: 1425–1427 (1943).

Gunsalis, I. C.: Butylene Glycol Production by the So-called Ford Strain of *Bac subtilis, Jour. Bact.,* **48**: 261–262 (1944).

en, A., and G. S. Walpole: Chemical Action of *Bacillus lactis aerogenes* (Esche-
ich) on Glucose and Mannitol: Production of 2:3-Butyleneglycol and Acetyl-
nethylcarbinol, *Proc. Roy. Soc. (London), Ser.* **B, 77**: 399–405 (1906).

R., and E. Isaacs: British Patent 483,989, Apr. 28, 1938.

son, M. J.: Determination of 2,3-Butylene Glycol in Fermentations, *Ind. Eng.
Chem., Anal. Ed.*, **16**: 626–627 (1944).

nelson, H.: Studies with *Bacillus polymyxa.* I. Some Factors Affecting the Fer-
nentation of Wheat by *Bacillus polymyxa, Can. Jour. Research,* **C, 22**: 235–240
1944).

–: Studies with *Bacillus polymyxa.* II. Bacteriophage of *Bacillus polymyxa* in
Relation to the 2,3-Butanediol Fermentation, *Can. Jour. Research,* **C, 22**: 241–
50 (1944).

–: Studies with *Bacillus polymyxa.* IV. Nitrogen Requirements in Relation to
,3-Butanediol Production from Starch, *Can. Jour. Research,* **C, 24**: 99–108
1946).

- and A. G. Lochhead: Studies with *Bacillus polymyxa.* III. Nutritional Re-
uirements, *Can. Jour. Research,* **C, 22**: 273–279 (1944).

all, A. R.: "The Biochemistry of the Production of 2,3-Butylene Glycol by
ermentation, doctoral thesis, Iowa State College, Ames, Iowa, 1934.

er, A. J., and M. A. Scheffer: Process for the Preparation of 2,3-Butylene-
lycol from Carbohydrates, U.S. Patent 1,899,156, Feb. 28, 1933; also Canadian
atent 296,070, Dec. 24, 1929.

nbach, J. J., E. R. Kooi, E. I. Fulmer, and L. A. Underkofler: Laboratory Con-
nuous Countercurrent Liquid—Liquid Extractor, *Ind. Eng. Chem., Anal. Ed.,*
5: 473–474 (1944).

E. R.: Production of the 2,3-Butanediols by the Fermentation of Starch, *Iowa
ate Coll. Jour. Sci.,* **21**(1): 36–37 (1946).

–, E. I. Fulmer, and L. A. Underkofler: Production of 2,3-Butanediol by Fer-
entation of Cornstarch, *Ind. Eng. Chem.,* **40**(8): 1440–1445 (1948).

gham, G. A., and G. A. Adams: Canadian Patent 435,716, July 9, 1946.

–, ———, and R. Y. Stanier: Production and Properties of 2,3-Butanediol.
Fermentation of Wheat Mashes by *Aerobacillus polymyxa, Can. Jour. Re-
arch,* **F, 23**: 48–71 (1945).

T. M., E. I. Fulmer, and L. A. Underkofler: The Fermentation of the Stereo-
meric 2,3-Butanediols by *Acetobacter suboxydans, Iowa State Coll. Jour. Sci.,*
: 359–367 (1944).

J. D., and A. Castagne: Production and Properties of 2,3-Butanediol. XIV.
tanediol Analysis for Process Control, *Can. Jour. Research,* **F, 24**: 311–319
946).

ann, A. J.: *Jour. Am. Oil Chemists' Soc.,* **22**: 31 (1945).

, S. A., and A. H. Auernheimer: Configuration of the 2,3-Butylene Glycols,
ur. Am. Chem. Soc.,* **66**: 792–796 (1944).

H. H. Geller, and E. C. Lathrop: Conversion of 2,3-Butylene Glycol to 1,3-
tadiene by Pyrolysis of Diacetate, *Ind. Eng. Chem.,* **37**: 877–884 (1945).

A. C.: Kinetics of the Catalytic Dehydrogenation of Alcohols and Glycols in
Gaseous State, *Can. Jour. Research,* **B, 23**: 49–69 (1945).

: Production and Properties of 2,3-Butanediol. IV. Purity of the Levorota-
ry 2,3-Butanediol Produced by *Aerobacillus polymyxa. Can. Jour. Research,*
, **23**: 10–16 (1945).

: Production and Properties of 2,3-Butanediol. XXIII. Condensation of the
meric 2,3-Butanediols with Ethyl Acetoacetate, *Can. Jour. Research,* **B, 25**:
3–429 (1947).

A. C. Blackwood, and G. A. Ledingham: A 2,3-Butanediol-Glycerol Fermen-
ion, *Science,* **101**: 245 (1945*a*).

———, and ———: Dissimilation of Glucose by *Bacillus subtilis* (Ford's
ain), *Can. Jour. Research,* **B, 23**: 290–296 (1945*b*).

———, F. M. Robertson, and G. A. Ledingham: Production and Properties
2,3-Butanediol. XVIII. Dissimilation of Glucose by *Serratia marcescens, Can.
ur. Research,* **B, 25**: 65–69 (1947).

———, V. C. Haskell, and F. J. Macdonald: Production and Properties of Butanediol. VI. Dehydration by Sulphuric Acid, *Can. Jour. Research,* **B, 23**: 289 (1945).

———, ———, and ———: Production and Properties of 2,3-Butanediol. XXI. lytic Cracking of Cyclic Acetals and Ketals Derived from 2,3-Butanediol, *Jour. Research,* **B, 25**: 266–271 (1947).

——— and F. J. Macdonald: Production and Properties of 2,3-Butanediol. Cyclic Acetals and Ketals Derived from Levo-2,3-Butanediol, *Can. Jour. search,* **B, 25**: 70–79 (1947).

N. V. Nederlandsche Gist en Spiritusfabriek: Dutch Patent 33,878, Nov. 10,

Olson, B. H.: "Production of 2,3-Butylene Glycol by *Aerobacter aerogenes,*" th University of Wisconsin, Madison, Wis., 1945.

——— and M. J. Johnson: The Production of 2,3-Butylene Glycol by *Aerob aerogenes* 199, *Jour. Bact.,* **55**(2): 209–222 (1948).

Othmer, D. F., W. S. Bergen, N. Schlechter, and P. F. Bruins: Liquid-Liquid Ex tion Data, Systems Used in Butadiene Manufacture from Butylene Glycol, *Eng. Chem.,* **37**: 890–894 (1945).

———, N. Schlechter, and W. A. Koszalka: Composition of Vapors from Boi Binary Solutions, Systems Used in Butadiene Manufacture from Butylene col, *Ind. Eng. Chem.,* **37**(9): 895–900 (1945).

Paretsky, D., H. G. Wood, and C. H. Werkman: Bacterial Oxidation of 2,3-Buty Glycol, *Jour. Bact.,* **44**(2): 257 (1942).

Pederson, C. S., and R. S. Breed: The Fermentation of Glucose by Organisms of Genus *Serratia, Jour. Bact.,* **16**: 163–185 (1928).

Perlman, D.: Production of 2,3-Butylene Glycol from Wood Hydrolyzates, *Ind. E Chem.,* **36**: 803–804 (1944).

Porter, R., C. S. McCleskey, and M. Levine: The Facultative Sporulating Bact Producing Gas from Lactose, *Jour. Bact.,* **33**: 163–183 (1937).

Robertson, F. M., and A. C. Neish: Production and Properties of 2,3-Butaned XXIV. The Cyclic Sulphates of *meso-* and *levo-*2,3-Butanediol, *Can. Jour. search,* **B, 25**: 491–493 (1947).

Rose, D.: Production and Properties of 2,3-Butanediol. XXII. The Effect of Aero Conditions on the *Aerobacillus* Fermentation, *Can. Jour. Research,* **F, 25**: 2 279 (1947).

——— and W. S. King: Production and Properties of 2,3-Butanediol. V. Small-sc Production Unit, *Can. Jour. Research,* **F, 23**: 79–89 (1945).

Scheffer, M. A.: "De suikervergisting door bacteriën der coligroep," thesis, De 1928.

———: Microbiological Preparation of 2,3-Butylene Glycol, U.S. Patent 2,064,3 Dec. 15, 1936.

Schniepp, L. E., J. W. Dunning, H. H. Geller, S. A. Morell, and E. C. Lathro Pilot-plant Conversion of 2,3-Butylene Glycol Diacetate to 1,3-Butadiene, *I Eng. Chem.,* **37**: 884–889 (1945).

Senkus, M.: Recovery of 2,3-Butanediol Produced by Fermentation, *Ind. E Chem.,* **38**: 913–916 (1946).

Silverman, M.: The Microbiological Formation of Acetylmethylcarbinol, *Iowa St Coll. Jour. Sci.,* **17**: 120–122 (1942).

——— and C. H. Werkman: The Formation of Acetylmethylcarbinol from Pyru Acid by a Bacterial Enzyme Preparation, *Jour. Biol. Chem.,* **138**: 35–48 (1941).

Slade, H. D., and C. H. Werkman: Assimilation of Acetic and Succinic Acids C taining Heavy Carbon by *Aerobacter Indologenes, Arch. Biochem.,* **2**: 97– (1943).

Slotter, R. L., and C. T. Langford: Wheat Starch Manufacture, *Ind. Eng. Che* **36**: 404–408 (1944).

Stahly, G. L., and C. H. Werkman: Origin and Relationship of Acetylmethylcarbi to 2:3-Butylene Glycol in Bacterial Fermentations, *Biochem. Jour.,* **36**: 575– (1942).

Stanier, R. Y., and G. A. Adams: The Nature of the *Aeromonas* Fermentati *Biochem. Jour.,* **38**: 168–171 (1944).

-, ———, and G. A. Ledingham: Production and Properties of 2,3-Butanediol. :. Strains of *Aerobacillus polymyxa* in Relation to Filterability and Butanediol roduction, *Can. Jour. Research,* **F, 23**: 72–78 (1945).

- and S. B. Fratkin: Studies on the Bacterial Oxidation of 2,3-Butanediol and elated Compounds, *Can. Jour. Research,* **B, 22**: 140–153 (1944).

naier, A. J., and C. L. Lovell: Clarification of Acid-hydrolyzed Mash and Beer r the Production of 2,3-Butanediol from Corn, *Ind. Eng. Chem.,* **38**: 721–724 1946).

son, E. L., J. A. Wheat, and J. D. Leslie: Production and Properties of 2,3-utanediol. XIII. Purification of By-product Ethanol, *Can. Jour. Research,* , **24**: 300–310 (1946).

kofler, L. A., and E. I. Fulmer: The Production of 2,3-Butylene Glycol by ermentation, *Wallerstein Labs. Communs.,* **11**(32): 41–51 (1948).

ve, T. H.: British Patent 315,263, July 10, 1928.

-: French Patent 676,876, June 19, 1929.

-: British Patent 335,280, June 25, 1929.

-: German Patent 572694, Mar. 25, 1933.

le, G. S.: The Action of *Bacillus lactis aerogenes* on Glucose and Mannitol. art II. The Investigation of the 2:3-Butanediol and the Acetylmethylcarbinol ormed; the Effect of Free Oxygen on Their Production; the Action of *B. lactis rogenes* on Fructose, *Proc. Roy. Soc. (London), Ser.* **B, 83**: 272–286 (1911).

G. E., O. G. Pettijohn, and R. D. Coghill: Production of 2,3-Butanediol from cid-hydrolyzed Starch, *Ind. Eng. Chem.,* **37**: 1189–1194 (1945).

-, ———, L. B. Lockwood and R. D. Coghill: Optical Isomers of 2,3-Butaneol Produced by Fermentation, *Jour. Am. Chem. Soc.,* **66**: 541–542 (1944).

-, L. J. Wickerham, O. G. Pettijohn, and L. B. Lockwood: Levo-2,3-Butylene lycol and a Method for Producing the Same, U.S. Patent 2,359,950, Oct. 10, 1944.

nan, C. H.: An Improved Technic for the Voges-Proskauer Test, *Jour. Bact.,* ): 121–125 (1930).

## CHAPTER 23

# THE ACETIC ACID BACTERIA AND SOME OF THEI BIOCHEMICAL ACTIVITIES

The acetic acid bacteria belong to the family Pseudomonadaceae. cells are rod-shaped, but elongated, filamentous, club-shaped, swolle branched forms may occur. They may be motile or nonmotile and do form endospores. The bacteria may secure energy by the oxidatio ethanol to acetic acid, by the oxidation of various sugars and other a hols, or by anaerobic dissimilations.

The following members of the genus *Acetobacter* are listed and scribed in "Bergey's Manual":

*Acetobacter aceti* (Kützing) Beijerinck (the type species)
*A. pasteurianus* (Hansen) Beijerinck
*A. kuetzingianus* (Hansen) Bergey et al.
*A. xylinum* (Brown) Bergey et al.
*A. oxydans* (Henneberg) Bergey et al.
*A. rancens* Beijerinck
*A. melanogenus* Beijerinck
*A. suboxydans* Kluyver and de Leeuw
*A. roseus*

Henneberg has described the five following species of acetic acid b teria, which are not listed in the seventh edition of "Bergey's Manua

*Bacterium schuetzenbachii* Henneberg
*Bact. xylinoides* Henneberg
*Bact. orleanense* Henneberg
*Bact. vini acetati* Henneberg
*Bact. curvum* Henneberg
*Bact. dihydroxyacetonicum* Virtanen and Bärlund

Acetic acid bacteria have been described by Pasteur (1868); Hans (1879); Brown (1886); Beijerinck (1899); Henneberg (1909); La (1914); Kluyver and de Leeuw (1924); Visser't Hooft (1925); Herma (1928, 1929, 1931); Vaughn (1942); Shimwell (1948); Walker and Kul (1949); Frateur (1950); Tošić and Walker (1950); and others.

Useful information on the characterization of the acetic acid bacte will be found in the foregoing references and also in the publications Tošić and Walker (1946), Walker and Tošić (1946), Leifson (1953), a Frateur et al. (1950).

**Classification.** There have been various classifications, none of the completely satisfactory, proposed for the acetic acid bacteria. The mo tant of these are the ones presented in "Bergey's Manual of minative Bacteriology" (refer to Vaughn, 1942) and by Frateur ).

the classification presented in "Bergey's Manual," species of the *Acetobacter* are divided into two large groups: (1) those which e acetic acid to carbon dioxide and water and (2) those which do idize acetic acid. The species capable of oxidizing acetic acid are ided further into two groups, depending on their ability to utilize nium salts (in Hoyer's solution) as the sole source of nitrogen. *ti* is capable of utilizing ammonium salts. Of those not able to ammonium salts, *A. xylinum* forms a thick, zoogloeal mat of cellu- n the surface of liquid media, while *A. rancens*, *A. pasteurianus*, . *kuetzingianus* do not form such a mat. Of the species which do idize acetic acid, *A. melanogenus* forms a dark brown to blackish nt in glucose media; *A. roseus* forms a pink to rose pigment in e media; and *A. suboxydans*, with an optimum temperature of 30 to and *A. oxydans*, with an optimum of 20 to 25°C., do not form nts.

eur (1950) proposed the division of the *Acetobacter* into four , as shown in Table 127.

BLE 127. CRITERIA FOR THE DIFFERENTIATION OF THE ACETOBACTER GROUPS *

| up | First criterion, catalase | Second criterion, oxidation of acetic acid on yeast agar containing 3% ethanol and 2% $CaCO_3$ | Third criterion, oxidation of calcium lactate with formation of $CaCO_3$ | Fourth criterion, ketogenic ability on oxidogram | Fifth criterion, production of gluconic acid from glucose |
|---|---|---|---|---|---|
| ans .. | 0 | + | + | 0 | 0 |
| s ..... | + | + | + | 0 | 0 and + |
| lans .. | + | + and + or 0 | + | from + to ++ | from + to ++ |
| ans .. | + | 0 | 0 | ++ | ++ |

rateur: *Cellule,* **53** (fasc. 3): 287 (1950).

species of *Acetobacter*, with varieties, were recognized by Frateur grouping. The *peroxydans* group contained *A. peroxydans* Visser't nd *A. paradoxum* nov. sp.; the *oxydans* group, *A. lovaniense* nov. scendens (Henneberg) Bergey et al., *A. rancens* (Beijerinck), and rieties of this species, namely, *filamentosum* nov. var., *turbidans* r., *saccharovorans* nov. var., *vini* nov. var., and *pasteurianum*; the *dans* group, *A. aceti* (Pasteur) Beijerinck, *A. aceti* var. *muciparum* ck nov. var., *A. xylinum* (Brown) Beijerinck, *A. xylinum* var.

*xylinoides* (Beijerinck) nov. var., *A. xylinum* var. *maltovorans* (B inck) nov. var., *A. mesoxydans* nov. sp., and three varieties o species, namely, *saccharovorans* nov. var., *lentum* nov. var., and *le saccharovorans* nov. var.; the *suboxydans* group, *A. suboxydans* (Kl and de Leeuw), and three varieties of this species namely, *mucip* (Kluyver and de Leeuw) nov. var., *biourgianum* (Kluyver an Leeuw) nov. var., and *hoyerianum* (Kluyver and de Leeuw) nov. *A. melanogenum* Beijerinck, *A. melanogenum* var. *maltovorans* (B inck) nov. var., and *A. melanogenum* var. *malto-saccharovorans* ( erinck) nov. var.

Leifson (1953) studied the flagellation of 30 strains of the g *Acetobacter*. As a result of his study, he proposed a division of the ent genus *Acetobacter* into two genera: *Acetobacter* and *Acetom* The *Acetobacter* would include only peritrichously flagellated (wit average wave length of 2.7 $\mu$) or nonflagellated species which oxidize tate and/or lactate to carbon dioxide and water, while the *Acetom* would include polar multitrichously flagellated (with an average length of 1.4 $\mu$) or nonflagellated species which do not oxidize aceta lactate to carbon dioxide and water and would be placed in the fa Pseudomonadaceae.

**Nutritional Requirements.** The *Acetobacter* requires certain vitar of the B complex in addition to a nitrogen source, a carbon source, basic minerals.

*Vitamins.* Underkofler et al. (1943) reported that *A. suboxy* required pantothenic, *p*-aminobenzoic, and nicotinic acids and that hy lyzed casein or a known mixture of amino acids furnished adeq organic nitrogen. Foda and Vaughn (1953) found that *A. melanoge A. oxydans,* and *A. rancens* required the same vitamins and that the two of these also required thiamin. Rao and Stokes (1953) confirmed requirements for strains of *A. suboxydans* and *A. melanogenus.* Rain and Mitson (1953) found that *A. capsulatum, A. gluconicum, A. tu dans,* and *A. viscosum* required pantothenate and nicotinate, whereas requirement for *p*-aminobenzoic acid was less marked when the perio incubation was extended.

The requirements of the *Acetobacter* for vitamins are generally satis by the use of yeast water, yeast extract, autolyzed yeast, malt extr malt sprouts, or corn-steep liquor.

*Nitrogen.* Amino acids appear to be suitable sources of nitrogen species of the *Acetobacter,* as was shown by Underkofler and associ for *A. suboxydans.* Foda and Vaughn (1953) observed that valine, leucine, alanine, cystine, histidine, and proline were required by *A. o dans* and *A. rancens* but that cultures of *A. melanogenus* required amino acids. Rainbow and Mitson showed that casein hydrolyzate w satisfactory source of nitrogen for *A. acidi-mucosum* (N.C.T.C. 64 *A. mobile* (N.C.T.C. 6428), *A. suboxydans* (N.C.T.C. 7069), *A. capsu tum* (N.C.T.C 4943), *A. gluconicum* (N.C.T.C. 4739), *A. turbid* (N.C.T.C. 6249), and *A. viscosum* (N.C.T.C. 6426). Hall et al. (19 reported that omission of alanine from the complete medium used by t ed in reduced growth of *A. acidi-mucosum*, *A. ascendens*, *A. capsula-* *A. pasteurianus*, *A. melanogenus*, and *A. suboxydans*.

e ability of an *Acetobacter* species to utilize or fail to utilize ammo- salts in Hoyer's medium as a source of nitrogen is an important in the classifications of Visser't Hooft (1925) and Vaughn (1942). ver, the ability to utilize ammonium salts as a source of nitrogen is widespread among *Acetobacter* species than was suspected at one Foda and Vaughn (1953) showed that strains of *A. melanogenus*, *ydans*, *A. rancens*, and *A. suboxydans* were able to utilize ammonium s the sole source of nitrogen when the required nutrients were sup- in the medium. Rao and Stokes (1953) reported that many strains *suboxydans* and *A. melanogenus* were able to utilize ammonium en for growth when the required growth factors and appropriate s of carbon and energy were available. Glucose, arabinose, manni- rbitol, and glycerol were suitable sources of carbon; ethanol, pyru- and lactate were unsuitable carbon sources. An unidentified factor in yeast extracts, tryptone, pork and beef livers, and other bio- l materials was necessary for the growth of these bacteria with ol, sodium lactate, and sodium pyruvate, both with ammonium e and with hydrolyzed casein.

l et al. (1953) reported that satisfactory growth of strains of *ti*, *A. acetigenum*, *A. acetosum* (three out of four strains tested), *etzingianum*, *A. mobile*, *A. orleanense*, *A. peroxydans*, *A. turbidans* out of two strains), *A. xylinoides*, and *A. xylinum* occurred in a glu- alts medium which contained 0.25 per cent weight per volume of ionium hydrogen phosphate as the only source of nitrogen and sugar oncentration of 2.6 per cent weight per volume.

*ization of Ethanol.* A number of scientists have reported that acid bacteria grow well in media containing ethanol only when cer- her substances are also present. Pasteur (1868) showed that *Myco-* (*A.*) *aceti* grew satisfactorily in ethanol-containing media only acetic acid was present also. Hoyer (1898) and Beijerinck (1898) ned this observation and demonstrated that *A. aceti* cannot grow in anol–mineral-salts–ammonium–nitrogen medium unless acetic acid, tate, or glucose is added. This was shown to be true of *A. rancens* *xylinum* also. According to Rao and Stokes (1953), *A. suboxydans*, . *melanogenus* behaved in a similar manner. Small amounts of e, fructose, mannitol, glycerol, or yeast autolyzate added to the l medium promoted growth of these species. It appears that sugar ted substances are required to initiate growth, after which ethanol e used as an additional source of carbon and energy.

**chemical Activities of the Acetobacter.** The biochemical activi- the *Acetobacter* consist mainly of aerobic and anaerobic dissimila- nd the synthesis of polysaccharides. The aerobic dissimilations are, he industrial viewpoint, most important, including the oxidative lation of sugars and alcohols. The oldest and best known of the tations brought about by acetic acid bacteria is that in which acid or vinegar is produced.

## VINEGAR

Vinegar may be defined as the condiment made from sugary or st materials by alcoholic and subsequent acetous fermentations. The literally signifies "sour wine," according to its derivation from the F (*vinaigre* = *vin*, "wine," plus *aigre*, "sour" or "sharp").

**Composition.** The composition of a vinegar will depend some on the nature of the raw material that has undergone alcoholic and tous fermentations. The conditions of manufacture, aging, and st will also influence the composition of the product. In a cider vinega example, one might find, besides at least 4 g. of acetic acid ($CH_3CO$ per 100 ml. of vinegar at 20°C., traces or small amounts of alc glycerol, esters, reducing sugars (as invert sugar), pentosans, salts other substances.

The Food and Drug Administration of the United States[1] has ado the following definitions and standards for vinegars:

*Vinegar, cider vinegar, apple vinegar.* The product made by the alco and subsequent acetous fermentations of the juice of apples. It contains, in cubic centimeters (20°C.), not less than 4 grams of acetic acid.

*Wine vinegar, grape vinegar.* The product made by the alcoholic and su quent acetous fermentations of the juice of grapes. It contains, in 100 c centimeters (20°C.), not less than 4 grams of acetic acid.

*Malt vinegar.* The product made by the alcoholic and subsequent ace fermentations, without distillation, of an infusion of barley malt or cereals w starch has been converted by malt. It contains, in 100 cubic centimeters (20° not less than 4 grams of acetic acid.

*Sugar vinegar.* The product made by the alcoholic and subsequent ace fermentations of sugar sirup, molasses, or refiners sirup. It contains, in 100 c centimeters (20°C.), not less than 4 grams of acetic acid.

*Glucose vinegar.* The product made by the alcoholic and subsequent ace fermentations of a solution of glucose, is dextrorotatory and contains, in 100 c centimeters (20°C.), not less than 4 grams of acetic acid.

*Spirit vinegar, distilled vinegar, grain vinegar.* The product made by acetous fermentation of dilute distilled alcohol. It contains, in 100 cubic ce meters (20°C.), not less than 4 grams of acetic acid.

**Historical.** Although vinegar has been known for thousands of ye its microbiological nature was not realized until a little more than years ago, when Kützing (1837) reported that the conversion of etha to acetic acid was brought about by living microorganisms. Fifteen ye earlier, Persoon had given the name *Mycoderma* to the film that for on liquids in which an acetic fermentation was taking place.

It remained for Pasteur (1868) to confirm Kützing's opinion and prove the physiological nature of the acetic acid fermentation. Past however, believed that the fermentation was caused by a single spe of bacteria, *M. aceti*. In 1878, Hansen showed that more than species of bacteria could bring about the souring of beer, i.e., the oxi f the ethanol to acetic acid. He isolated and named *Bacterium aceti* *act. pasteurianum*. At a later date he isolated *Bact. kützingianum*, a fourth species was described by Brown. About 1897 Henneberg d and reclassified the group and described several other species.

---

[1] *U.S. Dept. Agr., Service and Regulatory Announcements, Food Drug Admin.*, 2, Revision 5, Nov. 5, 1936.

**menclature.** The literature contains occasionally more than one for the same species of *Acetobacter*. For example, *A. aceti* has been ed to both as *M. aceti* and *Bact. aceti*. The system of nomenclature ed in "Bergey's Manual" will be used where possible in this chapter.

**neral Requirements for Manufacture.** In the manufacture of vine- veral factors are worthy of special consideration: the selection of icroorganism; the nature of the raw material; the concentration ethanol used, as well as that of the vinegar added at the start to y it; the amount of oxygen supplied; the nature of the supporting m; the temperature of the fermentation; aging and storage; clari- n; bottling and pasteurization; and the character and composition tanks, containers, and fixtures coming in contact with the vinegar the manufacturing process.

**ection of Microorganism.** Although there are large numbers of ia, as well as other microorganisms, that have the ability to produce acid in small amounts from various substrates, only relatively acteria possess the characteristics desired for vinegar production. *schuetzenbachii* or *Bact. curvum* may be used to produce acetic acid thyl alcohol in the quick vinegar process, while *Bact. orleanense* e used in either the quick vinegar or the Orleans process. *A. aceti*, *steurianus*, *A. xylinum*, *A. ascendens*, and *A. acetigenum* may be d from vinegar.

**ure of Raw Material.** Vinegar may be manufactured from almost oduct capable of yielding alcohol by fermentation. Fruits such as grapes, pears, peaches, plums, figs, and oranges; berries; honey; ontaining sirups; hydrolyzed starchy materials; beer; and wine rve as raw materials for vinegar manufacture.

and apple juice, or cider, are two of the best raw materials for r production—wine being used to a large extent in France, Italy, and Greece; cider in the United States. Vinegar prepared from popular, especially in England, while that from honey is consid- be very palatable.

obvious that the quality of the vinegar will depend in large part quality of the raw material used. Fruit should be clean, sound, the proper state of maturity; wine or alcoholic media should be lean, and free from preservatives. Cleanliness of the plant, equip- and surroundings are likewise indispensable for the production of a rade product.

**st Fermentation.** Before the acetic acid fermentation can take he sugar in the fruit juice or other sugar-containing medium must erted to alcohol by yeast fermentation. The yeast naturally pres- he fruit juice may bring about a successful spontaneous fermenta- it the manufacturer cannot rely on chance and should use a starter r to ensure a suitable fermentation. Although compressed yeast may be often used satisfactorily as a starter, the use of a selecte yeast, such as *S. cerevisiae* var. *ellipsoideus*, generally improv flavor of the final product. Fermentations may be carried out fav at 75 to 80°F. (23.9 to 26.7°C.). It is advisable to follow the course fermentation with hydrometers (Brix or Balling) that indicat approximate percentage of sugar present in the fermenting mash. the fermentation is complete, yeast, pulp, and other sediment sho removed from the medium by a process of settling. A storage per 2 to 3 weeks is usually allowed for the sedimentation, after whic clear medium is "racked" (drawn off), adjusted, if necessary, to the mum alcohol concentration, and acidified by the addition of some vinegar.

**Concentration of Alcohol.** Adjustment of the alcohol content medium may be necessary in order to ensure a successful ferment Alcohol in a concentration of 10 to 13 per cent is readily ferm When using alcohol concentrations of 14 per cent or greater, the zoo mat forms with difficulty and the alcohol is incompletely oxidized to acid. On the other hand, the use of too low concentrations may res the loss of vinegar, for, when the concentration of ethanol is less 1 or 2 per cent, esters and acetic acid are oxidized with the loss of a and flavor. Carbon dioxide and water are formed from acetic acid:

$$CH_3COOH + 2O_2 \rightarrow 2CO_2 + 2H_2O$$

With some species of acetic bacteria this action may occur even in no concentrations.

**Acidification.** The initial acidification is carried out with two ob in view: to inhibit the development of undesirable types of bacteria to supply desirable acetic-acid-producing bacteria for seed purposes. amount of vinegar added to the alcoholic medium will depend upor nature of the process, but usually 10 to 25 per cent by volume of st vinegar is considered to be sufficient. If the mix it to be permitted one passage through a generator, the initial acidity may be adjuste 3 to 3.5 per cent and sufficient alcoholic substrate added to yield a vin with a final acid strength of approximately 6 per cent (Cruess, 1958).

A medium should never be acidified before the alcoholic ferment is complete, because the sugar in the medium would be incompl converted to alcohol after the addition of acetic acid. Vinegars from incompletely fermented juices are usually low in acetic acid an poor quality.

**Oxygen.** Since the conversion of ethanol to acetic acid is prim an oxidation process, or a dehydrogenation in which atmospheric ox acts as the hydrogen acceptor, the success of the fermentation will de in no small part on the availability of large quantities of oxygen. following equations will illustrate the requirement for oxygen:

$$\underset{\text{Ethanol}}{CH_3CH_2OH} + \tfrac{1}{2}O_2 \rightarrow \underset{\text{Acetaldehyde}}{CH_3CHO} + H_2O$$

$$\underset{\text{Acetaldehyde}}{CH_3CHO} + \tfrac{1}{2}O_2 \rightarrow \underset{\text{Acetic acid}}{CH_3COOH}$$

mentation is to go on in casks or barrels they should be provided
n adequate number of holes above the surface of the liquid to ensure
ntiful oxygen supply. The openings should be screened to keep out
s, and the barrels should be only partly filled. When generators are
hey should be packed rather loosely with zoogloeal-supporting mate-
o permit sufficient ventilation in all parts of the generator.

nmercial production of vinegar and acetic acid by fermentation is
y carried out in large casks of proper design or in generators con-
ed in the form of a truncated cone provided at top and bottom with
ated scaffolds, and near the bottom with air inlets. The generator
cts as a stack or chimney in which a strong upward current of air
place, bringing the oxygen in contact with the bacterial film resident
surfaces of the supporting material for the film. (In the section on
ds of manufacture, the construction will be taken up in greater
)

**Supporting Medium.** The use of a satisfactory supporting
m has much to do with the duration and success of the acetic
ntation, since its purpose is to increase greatly the area or surface
d and thus accelerate the fermentation by virtue of the availability
rger oxygen supply.

ny manufacturers of vinegar during and before the time of Pasteur
to realize the necessity for an adequate oxygen supply and the use
pport for the film in containers where the zoogloeal mat was likely
disturbed periodically. Disturbed films sank and used up the nutri-
aterials anaerobically without producing acetic acid. Pasteur
d out defects in the processes and made suggestions about how they
e improved.

aft" or light wooden grating may be used as a support for the
al film in the cask or barrel. In the rapid fermentation process,
generators are used, the supporting medium is usually constructed
hwood shavings or chips, although rattan, wood charcoal, coke,
pomace, corncobs, excelsior, Berl saddles, brushwood, grape
or other materials that offer large surface areas may be substi-

ideal packing or supporting material should be effective in pro-
satisfactory acetification of the alcoholic substrate; should be non-
hould not impart odors, flavors, or color to the finished product;
not contribute iron, copper, sulfur, or other undesirable ions to
negar; should not pack, mat, or soften appreciably; should not
color from the substrate; should be durable; and should be readily
ble, preferably at a low cost.

xing materials differ in their relative values, and oftentimes avail-
and economy may result in the choice of a material not at the top
list. Beechwood shavings are generally regarded as being highly
ble but are not the lowest in cost. Coke may contain too much iron
ur to be satisfactory, and samples must therefore be analyzed
acceptance. Corncobs have given satisfactory results in the pro-
of wine and other vinegar, but they tend to soften and pack and

must be replaced generally after use for a season. Pumice stone co
sulfur compounds, although it is normally free from iron.

Allgeier et al. (1954) showed (in pilot-plant generators) that the
of distilled vinegar obtained with coke, rattan, and Berl saddles m
practically equal to those secured with beechwood shavings, pro
that the packing materials are properly prepared before use and
adequate nutrients are supplied to the infusion in order to maint
satisfactory bacterial film on the packing surfaces. Coke of 0.
0.25-in. diameter was washed with 10 per cent hydrochloric acid
water before use. The Berl saddles (the type used for distillation
umns) were composed of unglazed porcelain of 0.5-in. size. They we
56 lb. per cu. ft. and possessed a surface area of about 155 sq. ft. per c
They were soaked in 5 per cent hydrochloric acid for 72 hr., rinsed
times by alternately filling and emptying the generator, and then wa
in running water before use. Afterward they were soaked in 100-g
warm vinegar for 48 hr.

Allgeier and associates used S.D. 35A ethyl alcohol diluted with
water to provide a concentration of 9.0 to 9.2 g. of alcohol per 100 m
the feed for the generators. A concentrate of malt (Diamalt), dilute
15 g. per 100 ml., was fermented with yeast, filtered through No. 12 W
man paper, and used as a basic nutrient in the proportion of 25 ml.
2,000 ml. of diluted alcohol. The special nutrients added to the
included corn-steep water, autolyzed yeast, and malt extract. It is es
tial to add the special nutrients in minimum adequate amounts to pro
maximum acetification, for excess quantities may lead to slime forma
in the generators.

**Temperature Relations of the Acetic Acid Bacteria.** The ac
group of bacteria is characterized by very definite and peculiar temp
ture relations. At temperatures below 12 to 15°C. it grows slowly, and
cells are short but unusually broad. From 15 to 34°C. they appea
develop in what may be called the "normal" manner, growing rap
and developing chains of cells of varying number of units or eleme
In suitable media the walls become swollen and exhibit the early st
of zoogloea formation. At still higher temperatures (approximately
to 45°C.) long thread-like transparent filaments with no cross walls
with irregular bulging and occasional branching have been obser
This condition appears to be a pathological state induced by high te
perature, and if the culture is long maintained under these condition
may lose its power to function normally. A prompt return to tempe
tures of 15 to 34°C. will, however, result in the production of some c
of normal appearance and behavior.

This strange temperature effect is of importance in determining
most advantageous thermal conditions for active fermentation in vine
manufacture.

The exact temperature to be used will depend on the organism a
process being employed. In the United States a temperature of 80
85°F. (26.7 to 29.4°C.) is usually favorable for the acetic acid fermen
tion. In England, a higher temperature is sometimes used. The use

w a temperature favors a slow fermentation, while the use of too temperature favors the loss through evaporation of alcohol, acetic and the volatile substances important in the production of flavor roma.

**rage.** Previous to storage, the vinegar fermentation should be d to proceed until the vinegar has reached its maximum strength. this stage in the fermentation, when all the alcohol has been con- to acetic acid, the acetic acid bacteria or their enzymes will gradu- estroy the vinegar by oxidation, unless they are inhibited through on of oxygen or other means. Barrels or tanks, therefore, in which r is to be stored should be completely filled and then sealed to t access of air to the vinegar.

**ng.** Aging is a process that improves the flavor and clarity of r, especially vinegars made from wine or fruit juices. Esters are l, and the harsh flavor and odor of the fresh product disappear (see 7). Aging takes place during storage and may require a year or Alcohol vinegars, which are essentially dilute acetic acid solu- re not improved by aging.

**ification.** Some vinegars may be bottled without further treat- but most of them should be clarified first. Clarification may be l by filtration, using filter aids, or by fining. The former method is referred, however.

ne process of fining, a substance such as fish isinglass or bentonite nish clay is thoroughly mixed with the vinegar, the mixture is ted to stand until a clear vinegar appears, and finally the clarified r is drawn or siphoned off. Usually 1 oz. of fish isinglass, 0.5 lb. tonite, or 1 to 5 lb. of Spanish clay will be sufficient to clarify . of vinegar (Cruess and Joslyn, 1934).

**ing and Pasteurizing.** The vinegar should be properly aged, nd bright before it is bottled. Bottles should be completely filled tly capped or corked with treated corks to prevent access of air. ization is carried out by heating the bottles to a temperature of °C. (140 to 150°F.) for about 30 min. or until the temperature of gar within the bottles has reached at least 60°C. (140°F.). An ive method is to pasteurize the vinegar in bulk, cool it to 21.1°C. , and then bottle it.

ment of vinegar with silver ions by passing the vinegar between lectrodes, through which an electric current is passing, has been d to some extent during the past few years. The silver tends to development of the bacteria present in the vinegar by acting as a de and antiseptic.

**position of Containers and Equipment.** Since vinegar is corro- ntact with certain metals should be avoided in its manufacture, and distribution. Iron is readily attacked and eventually dis- nd may cause a clouding and browning of the vinegar (see the on the darkening of vinegar). Zinc may produce an undesirable the product and, in addition, form a poisonous zinc acetate. zed-iron equipment is therefore unsatisfactory on account of the zinc contained in it. Copper and brass should not be used as the likewise be attacked and affect the flavor of vinegar adversely.

Wood, aluminum, glass, hard rubber, and pressed paper may b satisfactorily in contact with vinegar, as may stainless steel.

**Methods of Manufacture.** Vinegar is made as a home indust simply providing an air supply and allowing barrels of cider or w ferment spontaneously. This does not always produce a high- product.

The commercial methods of manufacture include slow and rapid esses. A few typical illustrations of methods will follow.

*Orleans Process.* Of the slow processes, the Orleans, or "F method," is the oldest and also the best for the production of vinegars. Barrels of approximately 200-liter (52.8-gal.) capacit used as the containers in this process. Each barrel is filled about third full with a good grade of vinegar, which constitutes the start culture, and 10 to 15 liters of wine are added. At weekly interva 4 weeks, the same amount of wine is added to the barrel. When 5 w have passed, 10 to 15 liters of vinegar are withdrawn from the b which is now about one-half filled, and the same amount of wi introduced. The operation may be repeated, thus becoming a sl continuous process.

Air is admitted to the barrels through holes—one usually at each of the barrel, which is placed on its side—above the level of the vi medium. These holes are at least 1 in. in diameter and screened to vent the entrance of insects. Air may also be admitted through a bunghole likewise screened. The acetic acid bacteria form a thin on the surface of the solution, and this film later becomes quite thick gelatinous. This gelatinous zoogloeal mat, which contains very l numbers of bacteria, is known as the "mother of vinegar." Eventu unless supported on a "raft" or framework, it will sink to the botto the barrel and a new film will form.

Although vinegar of high grade is produced by this method, it slow and costly process that involves much attention. The films easily disturbed by the addition of the alcoholic medium and the w drawal of vinegar. If they sink, they use up the nutrient substances fail to produce acetic acid under anaerobic conditions. Pasteur m suggestions about how to improve this process. One such sugges involved the use of a support for the film.

*Modifications of the Orleans Process.* Most of the slow methods modifications of the old Orleans process. A light grating of wood may floated on the liquid medium to support the bacterial film and preven from breaking up and sinking. Another method to prevent submerge of the film is to equip the top bunghole with a funnel attached to a g tubing that leads to the bottom of the barrel. Alcoholic solutions be added with a minimum disturbing effect on the film. A glass tube r be attached through a bunghole in the bottom of the barrel to serve a gauge for measuring the level of the liquid in the barrel during fermen nd as a means for withdrawing finished vinegar without disturbing ogloeal mat.

*e Quick Vinegar Process.* The quick vinegar process, now largely is also known as the "German process." Boerhave discovered in arly part of the nineteenth century that, when wine was permitted ickle down through a tall receptacle containing loosely packed ce, vinegar was rapidly produced. Schutzenbach (1823) modified ethod of Boerhave by introducing other types of porous material in to obtain maximum contact of the organisms with air. The method by Schutzenbach is the basis for modern methods of manufacture the generator.

*nerators.* Generators are of various sizes and shapes. Some are in diameter and 20 ft. high. Some are 8 by 16 ft. or 4 by 8 ft. The ator is equipped with a false perforated bottom, through which air s. Some of the larger generators have a perforated shelf approxi- y halfway between the top and the bottom of the tank, which aids in rting the beechwood shavings or other material used to present a surface area for the acetic acid bacteria. The use of the perforated prevents crushing and matting of the shavings owing to the weight superimposed material. Near the top of the generator above the ngs there is a false top or perforated plate over which is arranged a ng sprinkler, or sparger, for producing a uniform distribution of the ar stock (vinegar plus alcohol-containing substrate) over the top e of the supporting material. In place of a sparger at the very top generator there may be located a tilting trough or other automatic e, which periodically dumps vinegar stock upon the distributing

vinegar stock may be passed through the same generator until the d acidity is obtained, or it may be passed through two or three sets erators connected in series, each with increasing acidity. The latter d of operation is known as "tandem operation."

enerator 10 ft. in diameter and 20 ft. high usually produces 80 to l. of distilled vinegar per day.

*Frings Method.* Vinegar may be manufactured by the Frings s (Hansen, 1935). This process, in fundamentals, is similar to the generator process, but it possesses several advantages, which will be oned later.

GS GENERATOR. Figure 70 shows a cross section of the Frings gen- . The generator consists of an airtight tank, which is usually 14 ft. meter and 15 ft. in height, and certain accessory equipment. The is superimposed on concrete beams, in such a manner that air may ate beneath the apparatus. Inside the tank, near the bottom, is a n grating, which supports beechwood shavings. These shavings are to within about 1.5 ft. of the top of the generator. Below the n grating at the bottom of the tank is the collection chamber of the tor. At the very bottom of this chamber is a cock or faucet through the finished product is withdrawn. Near the bottom of the tank,

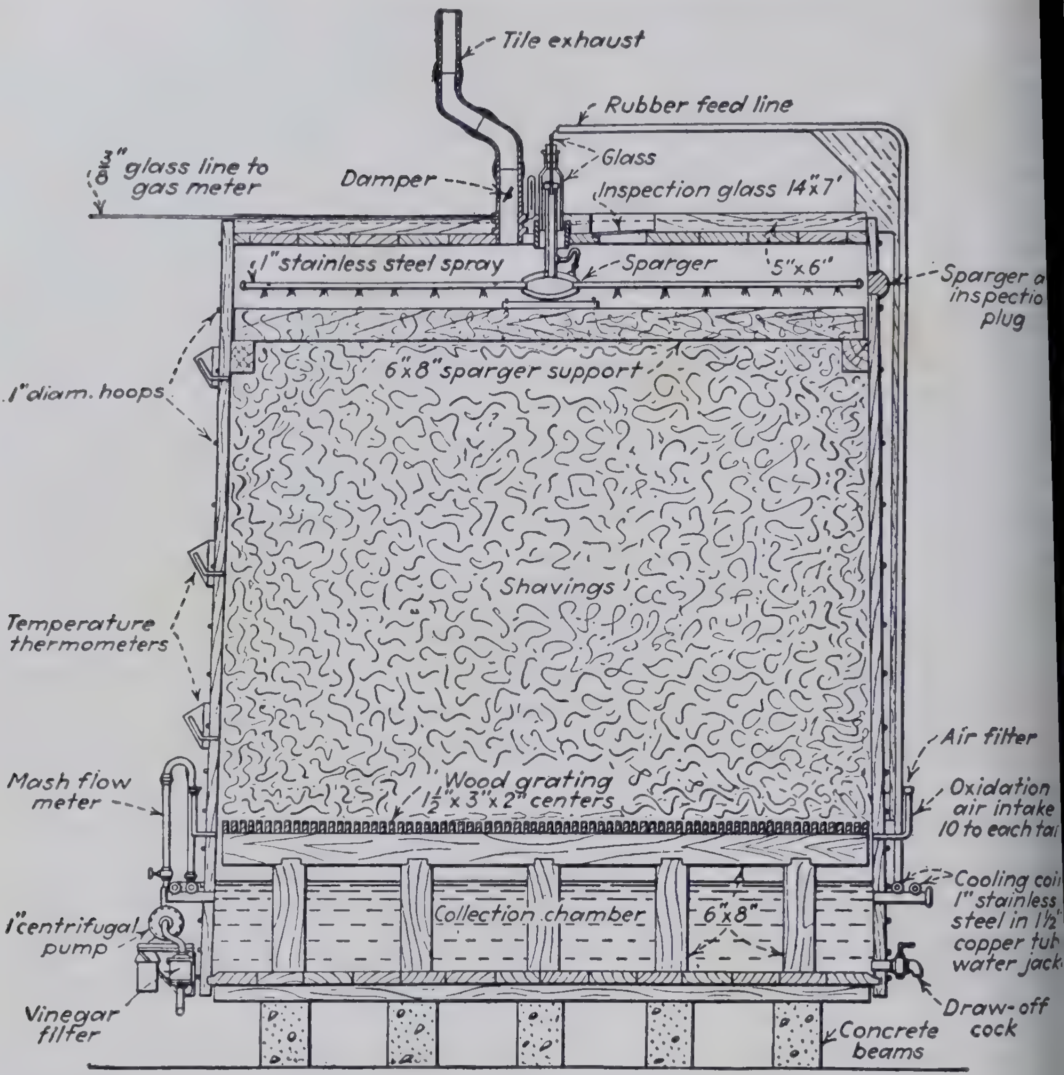

Tile exhaust
Rubber feed line
3/8" glass line to gas meter
Damper
Glass
Inspection glass 14"x7'
1" stainless steel spray
Sparger
5"x6"
Sparger a
inspectio
plug
6"x8" sparger support
1" diam. hoops
Shavings
Temperature thermometers
Air filter
Oxidation air intake 10 to each ta
Mash flow meter
Wood grating 1½"x3"x2" centers
Cooling coi
1" stainless
steel in 1½"
copper tub
water jack
1" centrifugal pump
Collection chamber
6"x8"
Draw-off cock
Vinegar filter
Concrete beams

Fig. 70. Cross section of the Frings generator. [*Courtesy of A. E. Hansen, Fo Inds.,* 7: 277 (1935).]

cooling coils are also located. The vinegar mix, which has passed do through the shavings, is circulated through an inner pipe made of stai less steel, or other acid-resistant substance, and is cooled by the passa of water through a surrounding outer pipe of copper. The mix is th forced by means of a centrifugal pump made of stainless steel from t cooling coils to the top of the tank through a rubber line. The mix pass from the rubber feed line through a glass connecting device to a sparg or distributing arm, constructed of stainless steel.

The oxygen supply of the generator is controlled by a damper, locat in a 4-in. vent constructed at the top of the tank. This vent constitut the air outlet of the generator. Air enters the generator through a seri of intakes (10) located around the tank near the level of the wooden grat These inlets contain air filters.

Thermometers are located at different points in the generator in ord to determine the temperatures in the different parts.

The generator contains a meter, attached near the pump, for measu ing the rate of flow of the mix or medium and also a meter for recordi

te of flow of the cooling water. A generator of the type just described
roduce approximately 30 times the quantity of white vinegar that
produced by a tank (4 by 8 ft.) of the noncirculating type.

IPOSITION OF THE MIX. A 2,500-gal. mix may contain 10.5 per cent
anol, 1 per cent of acetic acid, and 7 lb. of a special medium for
acid bacteria, known as "aceto-pep" (devised by Frings).

RATION OF THE GENERATOR. The mix is permitted to circulate
edly through the beechwood shavings until vinegar of the desired
th has been obtained. During the process heat is generated, and
quantities of oxygen are consumed. Since the tank is airtight,
for the air inlets and vent, the continued evolution of heat would
ce a temperature sufficiently high to inhibit the action of the acetic
bacteria. By passing the nutrient acid-alcohol mix through the
g coils, the temperature of the mix is carefully controlled. The
l mix is returned to the sparger and sprayed over the surface of the
ngs. It trickles down through the shavings, acetic acid being
d from the ethanol. It is again cooled and recirculated. This cycle
ated until the acetic acid content of the mix has become 105 grains.
y require 8 to 10 days for the conversion of the ethanol in a 2,500-
atch to acetic acid of this concentration. The vinegar is then with-
n, except for about 200 gal., which is left as food for the bacteria
s a primer for the pump. A new batch of mix is promptly run into
nerator.

ANTAGES OF THE PROCESS. This process has several advantages. It
e operated at a low cost, and it is easily controlled and relatively
e. Vinegars of higher acetic acid concentrations than those produced
her types of generators may be produced. The tank utilizes less
than that required by other types of generators for the production
same quantity of vinegar in the same time. Losses caused by
ation and by the presence of fumes in the room are avoided, for the
tor is airtight. The temperature of the room does not affect the
rature of the interior of the generator adversely. Owing to continu-
eration, there is little tendency for slime to form in the generator.

*gar Generator with Automatic Control.* A vinegar generator sys-
as been designed by Elmer (1939) in which automatic control is
ed and in which the efficiency of yield is high. The vinegar mix
y gravity from an elevated reservoir through a fluid course to the
tor and thence to a storage reservoir. The fluid course is provided
vessel for measuring the mix. A vertically adjustable float in the
ring vessel actuates a cock to cause the mix to flow in from the
ed reservoir. The mix is automatically discharged from the meas-
vessel to the generator through the agency of a valve electrically
ed by an electric circuit regulated by a suitable clock. The tem-
re and rate of flow of air through the generator are regulated. For
r details, consult the patent.

*kin Process.* Mackin (1947) patented a process and equipment
ducing high-grade vinegar without the use of shavings or a similar
t. The conditions of production may be accurately and continu-

ously maintained. Essentially the process consists of spraying a nut solution of dilute alcohol containing vinegar bacteria through th nozzles of a sparger located near the top of a confined space, allowin mixture to fall as a fine mist through air being turbulently circulat a countercurrent direction within the space, collecting the mixture the bottom of the confined space, cooling it to below 95°F., and retur it for reatomizing. The air used in the process is filtered before use. temperature is maintained between 68 and 95°F. by means of coo water. For further details of the process and for a description of equipment, the reader is referred to U.S. Patent 2,423,897, July 15, 1

*Revolving Generators.* Some vinegar is made by revolving genera These generators are essentially rotating drums or cylinders filled shavings. The acidified nutrient alcoholic substrate is added in quantity that the generator is approximately half filled (some dr hold 500 gal.). The cylinder is then caused to rotate slowly, possibly 1.5 r.p.h., until vinegar of the desired acidity has been obtained, a pro that may require about 3 weeks in some instances. Air, admitted thro inlets, supplies oxygen to the upper portion of the drum. The slow rat rotation causes the mix to become oxygenated. This method, accord to Cruess, is not too popular, probably on account of the expense invol in constructing the drums and in operating them and also owing to th complexity.

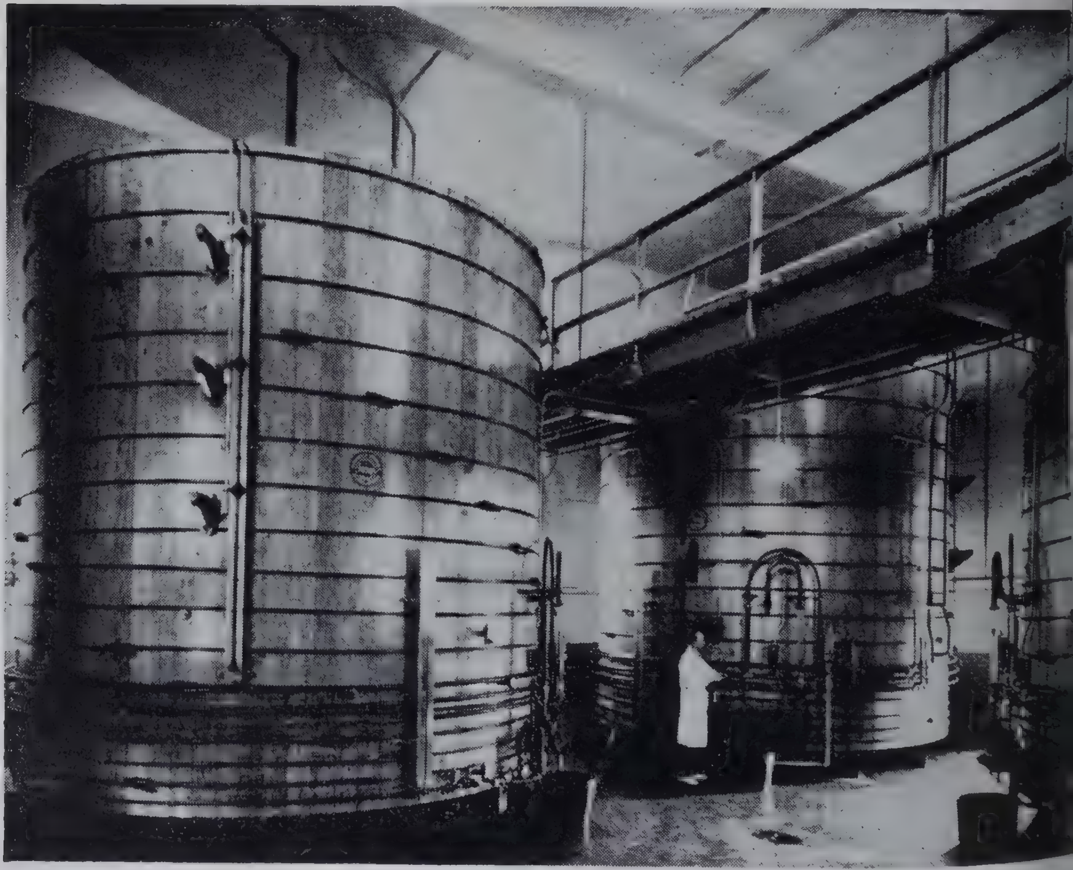


FIG. 71. Vinegar generators in air-conditioned buildings. [*Courtesy of L. E. Slat Food Eng.,* 25(7): 88 (1953).]

*odern Plant.* Slater (1953) described a vinegar plant of modernistic n capable of producing 20,000 gal. of vinegar a week. An air-itioned building, 50 ft. square and 40 ft. high, housed three Frings ators and a tank for premixing and diluting the charges for the ators (Fig. 71).

ch generator was about 18 ft. high and 17.5 ft. in diameter and had acity for 7,000 gal. of mix in the collection system located just the false bottom. There were provisions for automatic feed control, erature control, air control, analysis of the oxygen, and other res.

cated at the top of each generator was a stoneware receiver (with a ol box), which contained five solenoid-operated ball-seated poppet s for feed control (Fig. 72). These, when open, delivered the vinegar

2. Stoneware receiver containing poppet valves (on left) and control box elays for the poppet recirculating valves. [*Courtesy of L. E. Slater, Food Eng.,* 88 (1953).]

o a revolving sparger. One poppet valve remained open normally. peration of three others was governed by temperature controllers 73), located at three levels in each generator, which actuated relays e poppet recirculating valves. A second poppet valve opened when

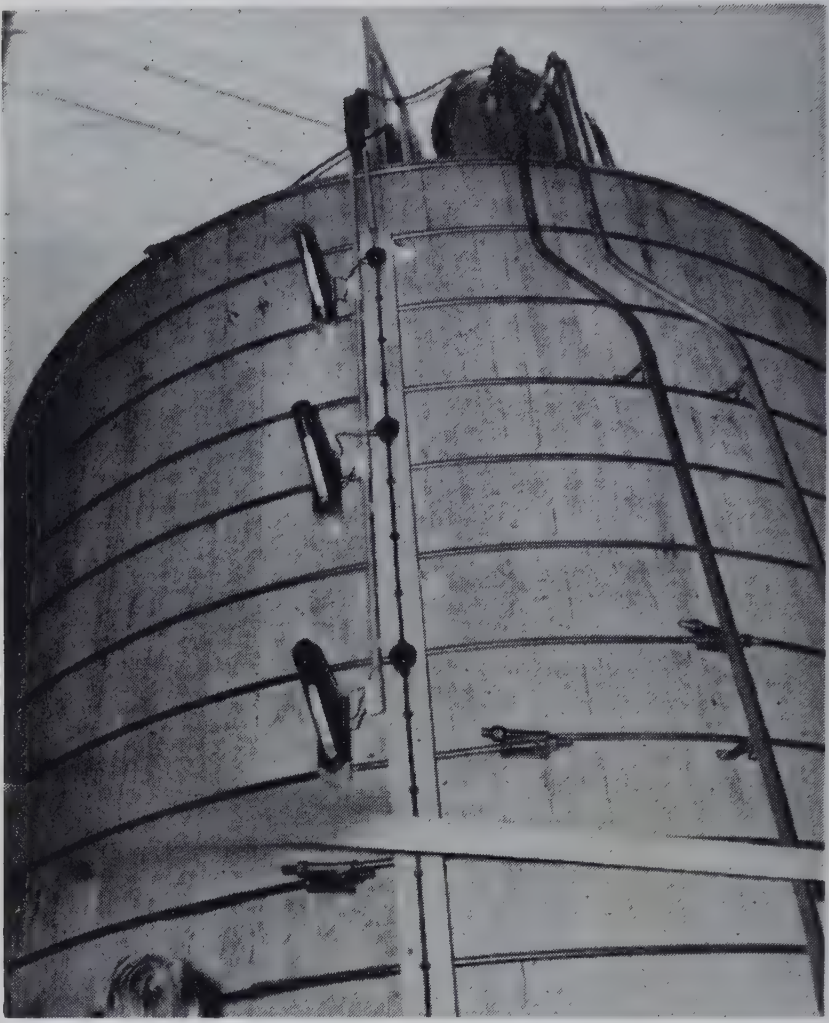


FIG. 73. View of generator. At the top right is the stoneware receiver. The devices shown on the front of the generator are part of a system for controllin temperature of the infusion. [*Courtesy of L. E. Slater, Food Eng.*, **25**(7): 88 (19

the temperature in the upper level went above 28°C.; a third, when temperature in the mid-level went above 31°C.; and a fourth, when temperature at the lower level exceeded 33°C. A fifth poppet remained closed normally but was opened for maximum flow. This sys of flow control was especially valuable during the start of operations

The temperature of the mix was controlled by (1) regulating the of its circulation, (2) cooling it during recirculation, and (3) forcing through the shavings. Well water was used, in a concentric pipe located on the exterior of the generator near the bottom (Fig. 74), to the mix. A temperature-control bulb, located in the pipe betw the stoneware receiver and the sparger wheel, caused cold wate be fed into the heat exchanger when required. Flow meters provide visual means for checking the rates of flow of the mix and the air. opening in the exhaust pipe on top of the generator was adjuste balance the air exhaust with the forced-air input.

A unit was considered to be operating at top efficiency when the ox in the exhaust air was 10 to 12 per cent, as demonstrated by the use o oxygen analyzer.

4. View of base of generator showing concentric tube heat exchanger and ters for gauging air flow and recirculation rate. [*Courtesy of L. E. Slater, Eng.*, **25**(7): 88 (1953).]

**merged Production of Vinegar.** Hromatka et al. (1949, 1950, 1953) reported on research carried out on the acetic acid fermenta- with special emphasis on the submerged fermentation. In 1949 roduced acetic acid from alcohol by submerged fermentation and that acid formation increased with time in an exponential function. reported that the same quantity of alcoholic substrate may be fer- d 30 times more rapidly by this process than by most modern indus- ethods.

matka and Ebner (1950) determined the efficiency of acetic acid ia on beechwood shavings. They found that oxygen uptake by bacteria was linear with time when the oxygen content of the air ore than 12 per cent. When the partial pressure of the oxygen was d to below the value corresponding to 12 per cent, the velocity of n uptake fell. The fermentation efficiency stopped when the oxygen 5 per cent in the gas phase. Bacterial metabolism was injured by creasing of the partial pressure of the oxygen. When beechwood gs inhabited with bacteria were flooded with static medium, dam- the cells owing to lack of oxygen occurred locally, which became singly irreversible with increased time of flooding. When inhabited vood shavings were flooded with a moving, air-saturated medium, no damage to cells occurred. Undisturbed fermentative action when the medium was moved and when the oxygen consum replaced by bubbling air through it.

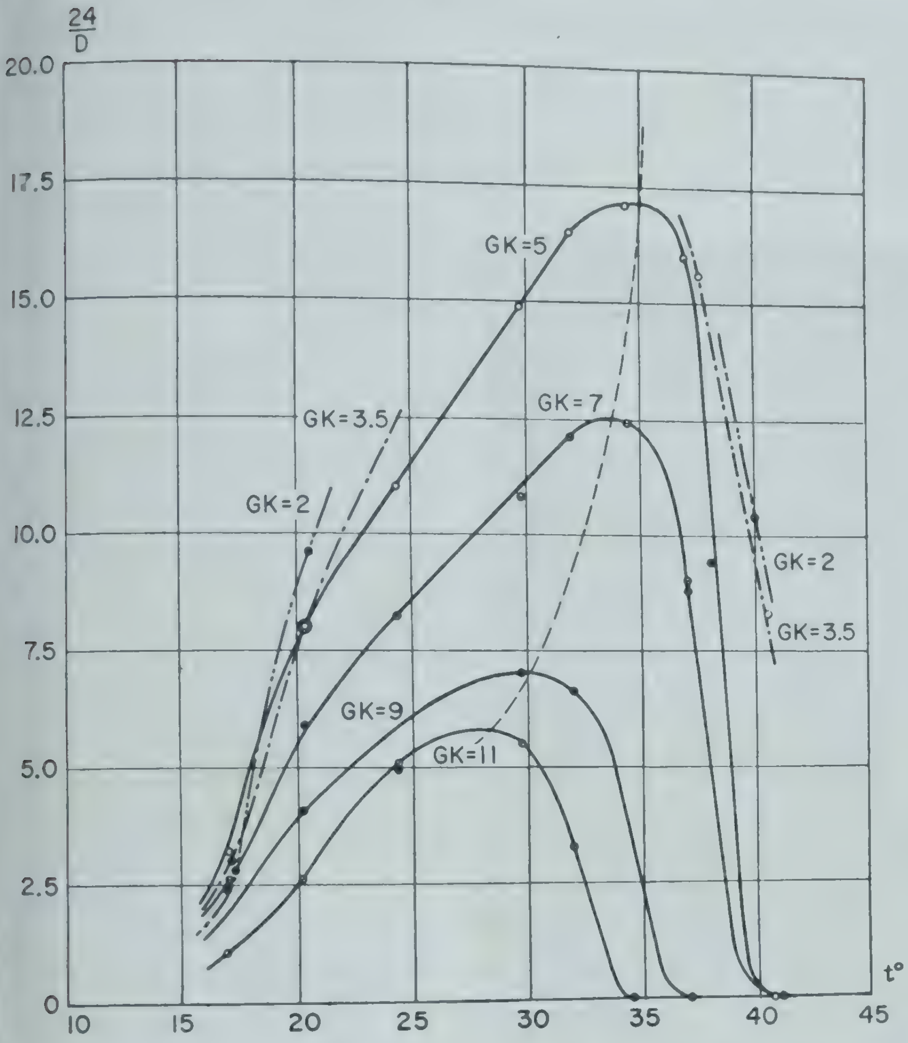
During a study of the effects of aeration on the submerged fe tion, Hromatka and Ebner (1951) made a number of significant o tions. It was found that the plot of the log of acid formation ($n$ was a straight line under optimum conditions but that the formati maximum and the subsequent decrease in log $n$ indicated an in effect. Maximum $n$-values were higher when the sizes of the air were smaller, based on the supply of equal amounts of air. The pure oxygen or a 60:40 mixture of oxygen and nitrogen retarded th merged vinegar fermentation, owing to the formation of a large c tration of acetaldehyde. The reproduction rate of the bacteria dec and finally stopped. The fermentation may actually cease unde conditions. Likewise, it was impossible to start a submerged fer tion when the bacteria were present in a low concentration and pur gen was used. When acid formation is very high, the use of a oxygen pressure higher than that normally found in air wou beneficial.

Hromatka, Ebner, and Czoklich (1951) carried out research conc the influence of a complete interruption of the aeration on the subm vinegar fermentation, using break periods of 15 sec. to 2 min. and a strate concentration ($GK$) of about 9.4 (for example, $GK = 9.11$ the acetic acid is 0.83 g. per 100 ml. and the alcohol 8.28 per ce volume). When the interruption of aeration was 60 sec. or longe damage was 100 per cent to the bacterial cells. At lower substrate centrations ($GK =$ about 5), the percentage of bacteria damaged much less, but even at this concentration breaks in aeration mu avoided. Damage to the cells in the absence of continuous aeration attributed to the toxic effects of alcohol and acetic acid.

Interruptions in aeration as long as 20.5 hr. did not destroy the ba completely where very dilute solutions (about those of isotonic tions) were employed. It is thus possible to inoculate substrates centrifuged cultures of bacteria, providing that the upper limit of not over about 8. Suspensions of bacteria concentrated by centrifug and those in very diluted solutions may be stored for months at refri tion temperature without aeration.

The influence of temperature and the total concentration of the strate ($GK$) on the submerged vinegar fermentation was studie Hromatka, Kastner, and Ebner (1953). They represented their re graphically (refer to Fig. 75) by plotting $24/D$ against temperatur different substrate concentrations ($GK$). The term $D$ denotes the within which $n$ (acid production) is reduplicated during the logarit phase of fermentation. The values for $D$ are influenced by tempera and by the total concentration ($GK$) of the substrate. The optimum perature of fermentation depends on the concentration of the subs and was found to be approximately inversely proportional.

Reference to the figure shows that the optimum temperature of fer



. Temperature relationships for submerged vinegar fermentation. [*Courtesy Iromatka, G. Kastner, and H. Ebner, Enzymologia,* 15 (fasc. 6): 22 (1953).]

was about 27°C. at a *GK* of 11, 30°C. at a *GK* of 9, 33°C. at a *GK* nd 35°C. at a *GK* of 5.

rder to avoid unnecessary losses of volatile materials at the higher ratures, the use of temperatures slightly below the optimal fermen- temperature is advocated.

as found that repeated changes in the temperature between 24.6 2°C. decreased the fermentation rate; however, a few such changes g the fermentation were harmless.

*Acetator.* This is a generator made by Heinrich Frings, of Bonn, any, which produces vinegars by submerged fermentation. It was ped after years of research by the latter concern and utilized details Hromatka-Ebner process and the Frings Moderator.

Acetator (Figs. 76 and 77) consists of a tank constructed of stain- el, wood, or lined steel and the necessary accessory equipment and ls. During operation, temperature, acidity, alcohol content, rate tion, and other factors are controlled. The acid and alcohol con- of the mix may be automatically indicated and the charging and

discharging of the tank automatically controlled. A flow she
in Fig. 78.

The Acetator may convert 20 to 160 gal. of absolute alcoh
and produce 66,000 to 528,000 gal. of 100-grain vinegar per ye
ing on the size of the unit. Tanks possess external diameters o
to 9 ft. 10 in. and a
heights of 9 ft. 10 in. to
and handle batch sizes
5,200 gal.

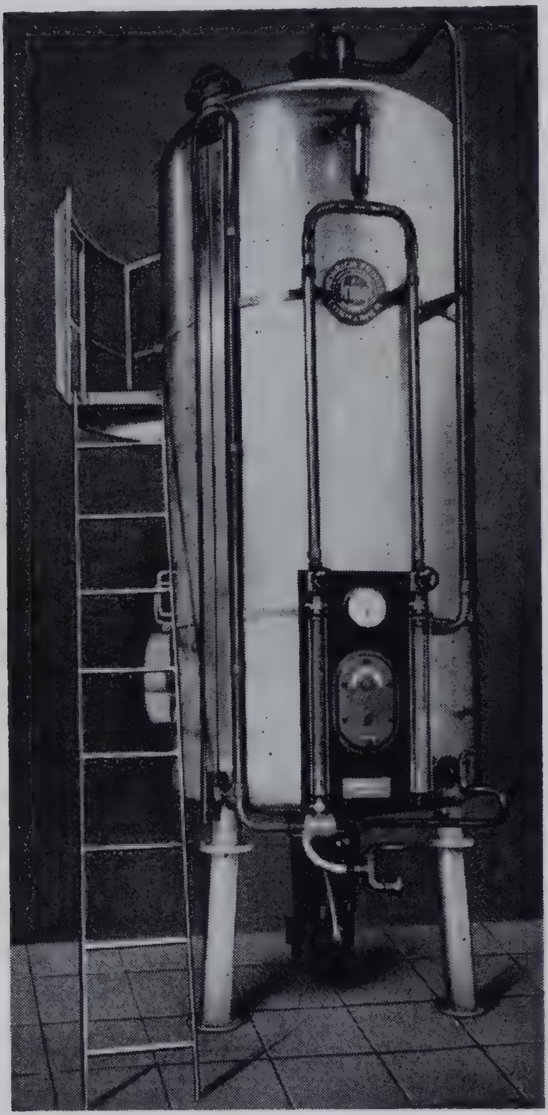


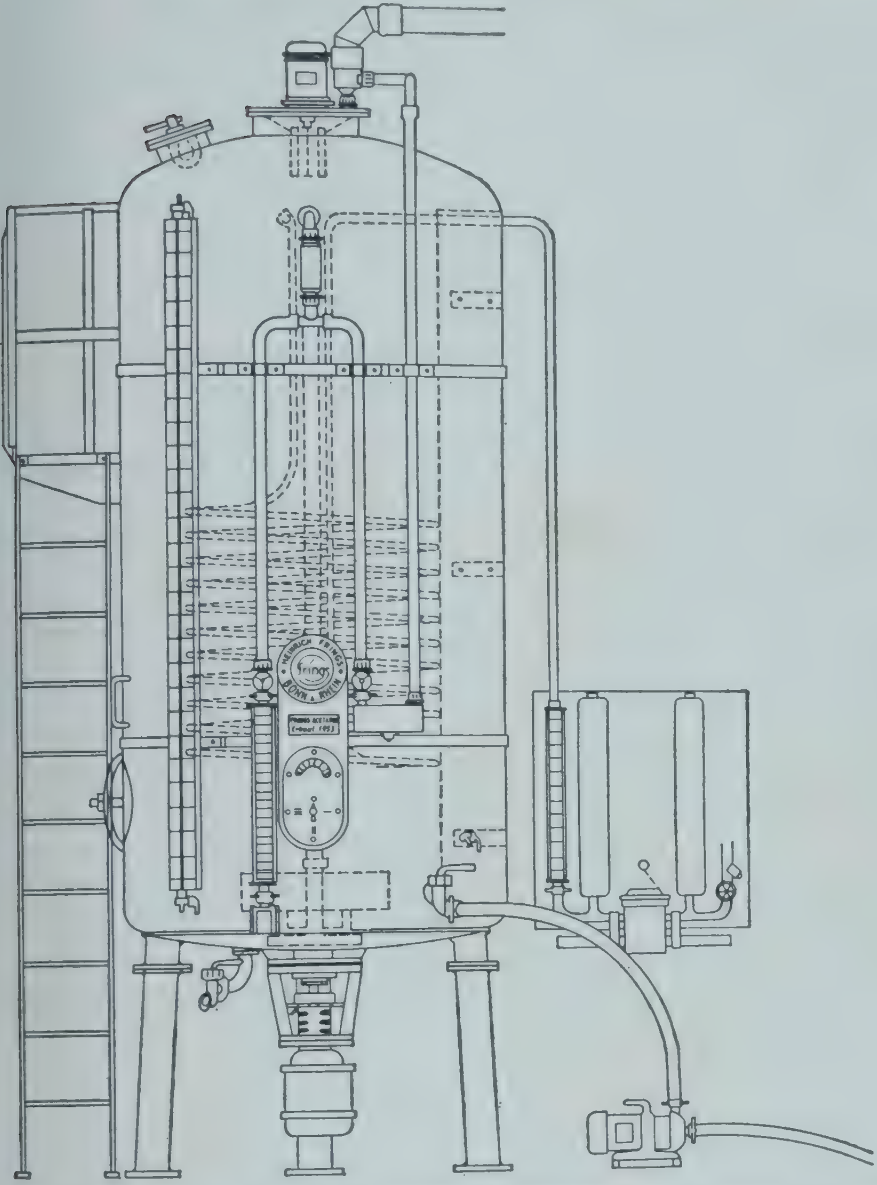
FIG. 76. Acetator. (*Courtesy of Heinrich Frings, Bonn, Germany.*)

Advantages claimed fo
merged process are (1)
(90 to 95 per cent of the
on the average); (2) high
(4 to 6 per cent or more
acid may be produced i
(3) good quality, for th
are present in large con
in the vinegar and im
quality of the product du
age; (4) simple utilizatio
materials, filtration and
cation being unnecessary;
zation of raw materials
alcohol (less than 6 per c
relatively high content of
(6) avoidance of the use o
sion vinegars when switch
one raw material to anoth
packing materials are not
easy adjustment of p
(may be in full operation
days without the use
amounts of starter vin
may be stopped easily);
savings in space owing
efficiency and yields;
creased economy owing to
ination of the clogging of
interruptions, etc.; and (10) simplified management (vinegar
removed every day or two).

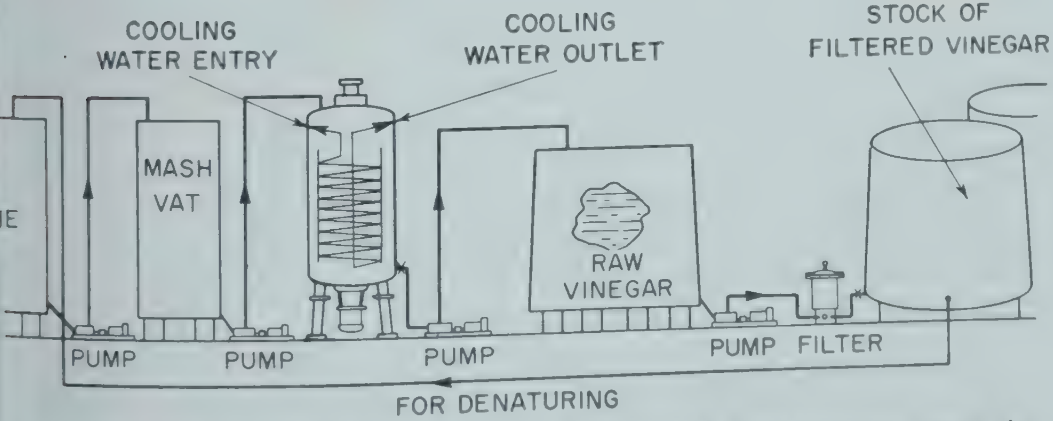
In view of the high oxidation rate of the submerged process, it
tial to maintain a residue of at least 0.3 per cent of alcohol in
prevent serious damage to the acetic acid bacteria.

Other details of the Acetator may be obtained from the
turer and by reference to the article by Mollenhauer (1956).

**Vinegar Defects.** Vinegar is subject to a number of defec
may be of chemical or biological origin. Such defects are often
cations acquired from the equipment. Iron, copper, tin, and


HEINRICH FRINGS
Frings
BONN a RHEIN

77. Detailed view of the Acetator. (*Courtesy of Heinrich Frings, Bonn,*
*any.*)


COOLING WATER ENTRY
COOLING WATER OUTLET
STOCK OF FILTERED VINEGAR
IE
MASH VAT
RAW VINEGAR
PUMP
PUMP
PUMP
PUMP
FILTER
FOR DENATURING

78. Flow sheet of vinegar production by submerged fermentation using the
tor. [*Courtesy of H. P. Mollenhauer, Food Manuf.*, 31(2): 47 (1956).]

sometimes cadmium, nickel, and bronze or brass alloys may be a of trouble.

*Iron.* Iron is usually dissolved by the vinegar as a result of co with a source of this metal. It may occur in vinegar as iron phospha iron tannates. Iron phosphates occur more commonly and caus iridescent haze which may possess a slightly bluish cast. Iron tann which may be blue, bluish-black, or black in color, may originate poorly treated cooperage, the equipment, or gelatin-tannin fines. ( narily about 100 p.p.m. of iron is required for the darkening of vin Aeration followed by fining usually removes the darkening cause iron tannates. Iron may accelerate the rancidity of fats when bro into contact with them.

*Copper.* Copper is a cause of clouding in wine vinegar, but a con tration of 25 p.p.m. apparently fails to cloud distilled vinegar. A con tration of 1 to 5 p.p.m. of copper may produce an off-color in cucum olives, peppers, and other chlorophyll-containing vegetables owin replacement of the magnesium in the chlorophyll with copper. A con of 10 p.p.m. produces intense changes. Thus, from the point of view preventing clouding of the vinegar and the discoloration of products which it is used, it is important to keep the copper content of vinega a minimum. Copper, like iron, may accelerate the rancidity of fats.

*Acetic Acid Bacteria.* Certain species of *Acetobacter* may cause def in vinegar. *A. aceti*, owing to its strong oxidizing abilities, may conv acetic acid to carbon dioxide and water. *A. xylinum* produces cellul material, which is more commonly known as "mother of vinegar."

*Oxidase.* Vinegar may occasionally become darkened owing to action of oxidase, an oxidizing enzyme. The latter may be destroyed pasteurizing the vinegar.

*Vinegar Eels.* Vinegar eels, i.e., nematode worms (*Anguillula ace* may be a source of considerable trouble in vinegar factories, especia when the fruit from which the cider or wine is made has not been ca fully controlled. They also gain access from dirt brought into the pl and from insects. They may attack the bacterial film and cause it sink and in some instances cause deterioration of the vinegar. They harmless to human beings but from an aesthetic or quality standpo are very objectionable in a product. Although quite small, about ⅛ long, they can readily be seen in a glass container by holding it befor strong source of light. In the factory they may be found around the ed of the surfaces of the liquid in barrels and in the generators. Th entrance can usually be prevented by keeping the plant in a high deg of cleanliness. Empty casks may be sulfured lightly to prevent th access. Once in the vinegar, they can be destroyed by heating the vine to a temperature of about 130°F. (54°C.) or by pasteurization, and th can be eliminated by filtration, using Filter-Cel, or by fining. Infect barrels, tanks, or generators may be treated with live steam.

*Mites.* Mites breed rapidly in the presence of warmth and moistu Cleanliness of a high order may be necessary to prevent mites fr appearing in an establishment. By placing a ring of turpentine or so viscid or repellent substance around each air hole in a cask or . their access may be prevented. In order to eliminate mites the ds used against eels may be employed. The room in which they are must be thoroughly cleaned and may be washed with an emulsion osene and water. Fumigating the room with sulfur helps sometimes. and hot water will destroy the mites.

*egar Flies.* Vinegar flies (species of *Drosophila*) breed in decayed fruit juices, and vinegar. By preventing these substances from being l about and by keeping the factory scrupulously clean their presence sually be avoided. The placing of screens over the windows and of the establishment and the use of fine screens over the holes in s in which the fermentation is taking place are very helpful in keept these and other flies.

*e Flowers.* "Wine flowers" is the term used by wine manufacto denote the whitish film, often much plicated, composed of yeastlls, which grows on the surface of wines or nutrient alcoholic solu- This film is sometimes called *M. vini.* The organisms making up m are strongly aerobic, grow very rapidly, and in the course of time idize many of the carbon-containing constituents to carbon dioxide ater. Flavor and alcohol are thus destroyed, while the solution es cloudy. Wine flowers can be prevented by storing the alcoholic n in completely filled and closed containers or tanks, or by adding of vinegar to 3 parts of the alcoholic solution.

**lds.** Under favorable conditions 50 to 55 parts of acetic acid may ained from 100 parts of sugar, or approximately 1.26 g. of acetic rom 1 g. of ethanol, according to Cruess. A portion of the sugar is ned in the production of substances other than ethanol and as food yeast. There is also a loss usually of some alcohol and acetic acid poration during the two fermentations.

**lem.** What yield of acetic acid can be obtained from 1 kg. of glucose, assuming ent efficiency in each of the conversion processes? How many grams and what (in liters) of air are theoretically required to convert the ethanol to acetic

*tion:*

$$\begin{array}{llll} \text{)} & (2 \times 46) & (2 \times 44) & \\ {}_2O_6 = & 2C_2H_5OH + & 2CO_2 & \text{(Gay-Lussac equation)} \\ \text{se} & \text{Ethanol} & \text{Carbon dioxide} & \end{array}$$

$$\begin{array}{lll} 46) & (2 \times 32) & (2 \times 60) \\ I_5OH + & 2O_2 = & 2CH_3COOH + 2H_2O \\ \text{anol} & \text{Oxygen} & \text{Acetic acid} \end{array}$$

oretically, 1 mole of glucose (180 g.) would yield 2 moles, or 120 g., of acid, which represents 2 parts of acetic acid from 3 parts of glucose; f glucose would yield (1,000/180) × 120 g. = 667 g. of acetic acid. ming 90 per cent efficiency in both reactions (1) and (2), the yield ic acid would be 667 × 0.9 × 0.9 = 540 g. This represents a yield arts of acetic acid from 100 parts of glucose.

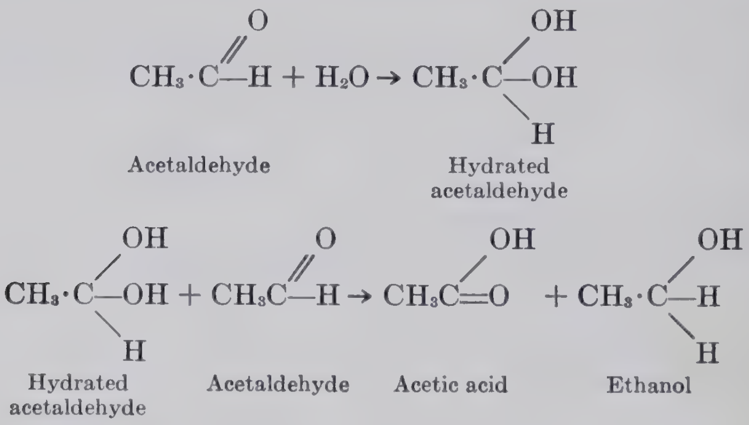
n Eq. (2), 92 g. of ethanol would require 64 g. of oxygen for conversion to acetic acid. Assuming 90 per cent efficiency in Eq. 460 g. of ethanol [1000 × (92/180) × 0.9], would theoretically re (460/92) × 64 = 320 g. of oxygen = 224 liters. Since air is app mately one-fifth oxygen by volume, the quantity of air required to vert 1 kg. of glucose to acetic acid would be 1,600 g., or 1,120 liters a matter of fact not all the oxygen in the air becomes fixed by the teria, and several times this volume should be available.

**Grains Strength.** The term "grains strength" is commonly use express the concentration of the acetic acid in a vinegar. One-g vinegar contains 0.1 g. of acetic acid in 100 ml. at 20°C. (1 mg. per Vinegar containing 4 g. of acetic acid per 100 ml. at 20°C., approxima 4 per cent, is of 40 grains strength. In other words, the grains stre is 10 times the acetic acid content in percentage.

**Uses of Vinegar.** Vinegar is used for several purposes in connec with food: as a condiment for direct use on the table or on a commer scale in the manufacture of mayonnaise, French dressing, pickles, relis catsup, prepared mustard, and horse-radish; for preventing mold gro in bread; and for other purposes. It may be used as an antise (McCulloch, 1945).

**Mechanism of the Acetic Acid Fermentation.** In the acetic acid mentation, acetaldehyde is an established intermediate product, hav been first shown to be such by Hoyer (1899). Its fixation can be dem strated with neutral calcium sulfite.

Under anaerobic conditions 1 molecule of acetaldehyde may act the hydrogen acceptor for a second hydrated molecule of the same s stance. The result is a Cannizzaro reaction in which 1 molecule of etha and 1 molecule of acetic acid are produced from 2 molecules of acetal hyde, the ethanol being formed by hydrogenation of acetaldehyde a the acetic acid by dehydrogenation of the hydrated acetaldehyde:



$$CH_3\cdot CHO + H_2O \rightarrow CH_3\cdot CH(OH)_2$$

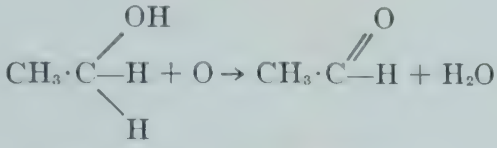
$$CH_3\cdot CH(OH)_2 + CH_3CHO \rightarrow CH_3C(OH){=}O + CH_3\cdot CH_2OH$$

Neuberg and Windisch are of the opinion that ethanol is aerobica transformed to acetaldehyde, which in turn is dismutated, in the mann illustrated above, to equimolar quantities of ethanol and acetic ac Alternate oxidation and dismutation follow until all the ethanol is co verted to acetic acid.

Neuberg and Windisch (1925) showed that *A. ascendens*, *A. pasteu*

and *A. xylinum* were able to dismutate acetaldehyde to equimolar
tities of acetic acid and ethanol anaerobically. They likewise dem-
ated that other aldehydes could be converted in a similar manner
eir corresponding alcohols and acids. Wieland and Bertho (1928)
Molinari (1929) have shown that similar reactions may take place
r anaerobic conditions.

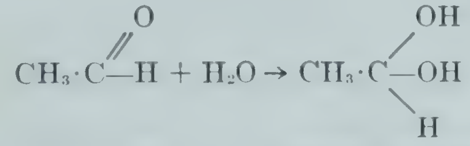
seems most likely, according to Butlin (1936), that in the normal
acid fermentation acetaldehyde is dehydrogenated to acetic acid.
en acts as the hydrogen acceptor in the conversion of alcohol to
ldehyde (a catalytic dehydrogenation):



$$CH_3{\cdot}C(OH)(H){-}H + O \rightarrow CH_3{\cdot}C({=}O){-}H + H_2O$$

Ethanol Acetaldehyde

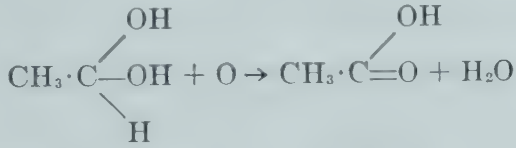
taldehyde is hydrated:



$$CH_3{\cdot}C({=}O){-}H + H_2O \rightarrow CH_3{\cdot}C(OH)(H){-}OH$$

Acetaldehyde Hydrated acetaldehyde

o of the hydrogen atoms of the hydrated acetaldehyde are activated
lonated to oxygen, the hydrogen acceptor:



$$CH_3{\cdot}C(OH)(H){-}OH + O \rightarrow CH_3{\cdot}C(OH){=}O + H_2O$$

Hydrated acetaldehyde Acetic acid

en conditions become favorable, some acetaldehyde may be con-
l to acetic acid by dismutation. In this case acetaldehyde becomes
drogen acceptor as well as the donator.

stances other than oxygen and acetaldehyde may act as hydrogen
tors, according to Wieland and Bertho. Methylene blue and benzo-
ne are two such substances.

## SOME OTHER FERMENTATIONS

e oxidations brought about by various species of the genus *Aceto-*
*r* are of particular significance, since some of the compounds, such
toses and keto acids, formed from suitable substrates by these bac-
are prepared with considerable difficulty by purely chemical
ods.

cies of the genus *Acetobacter* vary in their ability to oxidize or
rogenate various substances. Some species, for example *A. rancens*,
e a substrate to a high degree, sometimes forming carbon dioxide

and water as the principal end products. Such bacteria, obviously, have no industrial value. Other species of the genus *Acetobacter* bring about the incomplete oxidation of a substrate and, accordingly, may be of much importance.

*A. suboxydans* is a species well adapted for industrial use, for it generally brings about the incomplete oxidation of sugars, alcohols, and acids even when a liberal supply of oxygen is available, as is essential for a rapid dehydrogenation of the substrate. Butlin (1936, 1938) and Kluyver and Boezaardt (1938) have demonstrated, however, that the cells present in young cultures of *A. suboxydans* may produce some carbon dioxide from glucose.

*A. xylinum*, the sorbose bacterium, produces incomplete oxidation of substrates also, but in the presence of large quantities of oxygen may oxidize the substrates completely, forming carbon dioxide and water.

**Methods of Production.** The production of oxidized products by the acetic acid bacteria, particularly *A. suboxydans*, may be carried out by surface-culture or submerged-culture methods. Illustrations of both procedures are presented on the following pages.

Fulmer and Underkofler (1947) reported that the optimum conditions for the production of polyhydric alcohols with *A. suboxydans* by surface-culture methods were as follows: a pH of 6.1 (range of 5.1 to 6.8); a temperature of 28°C. (range of 25 to 30°C.); a yeast-extract (Difco) concentration in the medium of 0.5 per cent; and a surface/volume (square centimeters per cubic centimeter of medium) ratio of about 1.195. They stated that sorbitol may be fermented in concentrations up to and including 35 per cent; mannitol, in concentrations up to and including 25 per cent; glycerol, in concentrations not exceeding 6 per cent; and erythritol, in concentrations not exceeding 4.5 per cent.

**Nutrients of A. suboxydans.** The carbon is supplied as a polyhydric alcohol or other compound. Hydrolyzed casein or a known mixture of amino acids are satisfactory sources of organic nitrogen. Mineral salts are necessary to supply the usual elements not found in the other ingredients. Pantothenic acid, *p*-aminobenzoic acid, and nicotinic acid are required as growth substances (Underkofler et al., 1943).

Yeast extract or corn-steep liquor is generally used to supply the growth requirements of *A. suboxydans;* hence it is not necessary to add growth substances, salts, or mixtures of amino acids. Fulmer, Bantz, and Underkofler (1944) found that an acid-alfalfa extract supported growth of *A. suboxydans* satisfactorily but that it was somewhat inferior to yeast extract for this purpose. However, the yields of keto-compounds from glycerol, sorbitol, and 2,3-butylene glycol were of equal magnitude whether prepared with yeast extract or alfalfa extract.

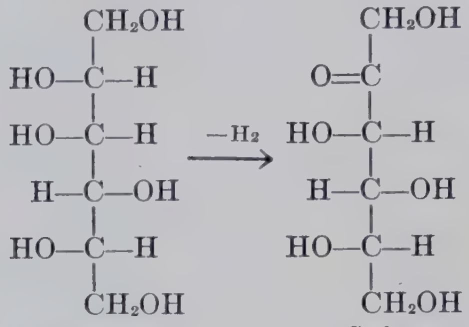
**Some Products Formed by A. suboxydans.** In Table 128 are shown some of the products formed by *A. suboxydans* as the result of the oxidation of suitable substrates. These products are in most instances also produced by other species of the genus *Acetobacter.*

TABLE 128. SOME SUBSTRATES OXIDIZED BY *Acetobacter suboxydans* AND THE PRODUCTS FORMED

| Substrate | Product formed |
|---|---|
| $CH_2OH$<br>HOCH<br>HOCH<br>HCOH<br>HCOH<br>HOCH<br>$CH_2OH$<br>Perseitol | $CH_2OH$<br>$O{=}C$<br>HOCH<br>HCOH<br>HCOH<br>HOCH<br>$CH_2OH$<br>Perseulose |
| CHO<br>HCOH<br>HOCH<br>HCOH<br>HCOH<br>$CH_2OH$<br>D-Glucose | COOH<br>HCOH<br>HOCH<br>HCOH<br>HCOH<br>$CH_2OH$<br>D-Gluconic acid |
| COOH<br>HCOH<br>HOCH<br>HCOH<br>HCOH<br>$CH_2OH$<br>D-Gluconic acid | COOH<br>HCOH<br>HOCH<br>HCOH<br>$C{=}O$<br>$CH_2OH$<br>D-5-Ketogluconic acid |
| COOH<br>HCOH<br>HOCH<br>HCOH<br>HCOH<br>$CH_2OH$<br>D-Gluconic acid | COOH<br>$C{=}O$<br>HOCH<br>HCOH<br>HCOH<br>$CH_2OH$<br>D-2-Ketogluconic acid |
| $CH_2OH$<br>HCOH<br>HOCH<br>HCOH<br>HCOH<br>$CH_2OH$<br>D-Sorbitol | $CH_2OH$<br>HCOH<br>HOCH<br>HCOH<br>$C{=}O$<br>$CH_2OH$<br>L-Sorbose |
| $CH_2OH$<br>HOCH<br>HOCH<br>HCOH<br>HCOH<br>$CH_2OH$<br>D-Mannitol | $CH_2OH$<br>$O{=}C$<br>HOCH<br>HCOH<br>HCOH<br>$CH_2OH$<br>D-Fructose |
| $CH_2OH$<br>HCOH<br>HCOH<br>HCOH<br>$CH_2OH$<br>*i*-Adonitol | $CH_2OH$<br>HCOH<br>HCOH<br>$C{=}O$<br>$CH_2OH$<br>"Adoninulose" |
| $CH_2OH$<br>HOCH<br>HOCH<br>$CH_2OH$<br>Erythritol | $CH_2OH$<br>$O{=}C$<br>HOCH<br>$CH_2OH$<br>L-Erythrulose |
| $CH_3$<br>HCOH<br>HCOH<br>$CH_3$<br>2,3-Butylene glycol | $CH_3$<br>HCOH<br>$C{=}O$<br>$CH_3$<br>Acetylmethylcarbinol |
| $CH_2OH$<br>HCOH<br>$CH_3$<br>α-Propylene glycol | $CH_2OH$<br>$C{=}O$<br>$CH_3$<br>Acetol |
| $CH_2OH$<br>HCOH<br>$CH_2OH$<br>Glycerol | $CH_2OH$<br>$C{=}O$<br>$CH_2OH$<br>Dihydroxyacetone |
| $CH_2OH$<br>$CH_2OH$<br>Ethylene glycol | $CH_2OH$<br>COOH<br>Glycollic acid |

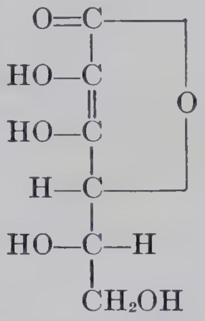
## THE SORBOSE FERMENTATION

L-Sorbose is a compound of especial interest on account of it the synthesis of vitamin C. By chemical means, D-sorbitol is pr from dextrose; by biological dehydrogenation, D-sorbitol is conve L-sorbose:



D-Sorbitol L-Sorbose

The relation of L-sorbose to vitamin C (L-ascorbic acid) can be trated by showing the structural formula of this vitamin as assign Haworth and others:



L-Ascorbic acid
(Vitamin C)

It has been known since the latter part of the nineteenth century certain oxidative bacteria have the ability to produce L-sorbose D-sorbitol. Bertrand, in 1896, published the results of some of hi searches with the sorbose bacterium, which was discovered by him, known as *Acetobacter xylinum.* The organism with which he worked called the "sorbose bacterium" because it produced sorbose from sorbitol present in the berries of the mountain ash (*Sorbus aucupari*

In general, species of the genus *Acetobacter* may be used to pro sorbose from sorbitol by oxidation. The most important of these *A. suboxydans, A. xylinum, A. rancens, A. aceti, A. melanogenus, A. conicum,* and *A. xylinoides.*

Sorbose may be prepared from sorbitol by two different metho fermentation: by surface growth and by submerged growths. In respect and in other ways this fermentation is similar to the gluconic fermentation.

**Surface Growth.** In some of the earlier researches, sorbitol in centrations of 2 to 6 per cent was used, the fermentations requiring ys for completion. Fulmer and his associates (1936) devised a d of fermentation in which sorbitol in concentrations up to and ing 35 per cent was readily fermented by *A. suboxydans* with high ields. Yeast extract was used in the fermentation mashes as a nt in a concentration of 0.5 g. per 100 ml. The rate of sorbose for- ı decreased with an increasing concentration of sorbitol, but the tage of final yield, based on the sorbitol originally present, varied tle over the range of concentrations from 5 to 35 per cent. A 40 nt concentration of sorbitol markedly reduced the activity of *A.* *ydans.* The temperature range of 25 to 30°C. was optimum for the ıtation.

ratio of surface to volume (square centimeters of area per 1 ml. of e) was important in this fermentation. The final yield, likewise e of sorbose formation, increased (within limits) with an increasing e/volume ratio.

ds of over 80 per cent of sorbose were obtained from sorbitol by regoing procedure. If the bacterial films or mats were disturbed flasks, the yields of sorbose were reduced.

**merged Growth.** Production of sorbose from sorbitol by sub- l growths in rotary drum fermentors has been successfully carried Wells, Stubbs, Lockwood, and Roe (1937) of the U.S. Department riculture. The procedure used by these men was similar to that ved in the drum method for gluconic acid production (refer to 29). Good results were obtained when the concentration of sorbitol ) per cent, the air flow 1,800 ml. per min., the gauge pressure 30 lb. . in., and the drum rotation 13 r.p.m. Fermentations were carried 30°C., using yeast extract in the proportion of 5 g. per liter as the lded nutrient material.

ABLE 129. THE EFFECT OF CONCENTRATION OF D-SORBITOL ON PRODUCTION OF L-SORBOSE BY SUBMERGED GROWTHS OF *Acetobacter suboxydans* *

v. 1,800 ml. per min.; r.p.m., 13; gauge pressure, 30 lb. per sq. in. (2.11 kg. per sq. cm.)]

| concen- per cent | Fermentation period, hr. | Total sorbitol available, g. | Sorbose produced | |
|---|---|---|---|---|
| | | | g. | Per cent by weight |
| 0.1 | 16.5 | 332 | 307 | 92.5 |
| 4.5 | 24 | 453 | 423 | 93.4 |
| 9.8 | 45† | 632 | 617 | 97.6 |
| ).3 | 69 | 975 | 194 | 19.9 |

. Wells, J. J. Stubbs, L. B. Lockwood, and E. T. Roe, *Ind. Eng. Chem.*, 29: 37). Copyright 1937 by the American Chemical Society and reprinted by on of the copyright owner.

nentation practically completed in 33 hr.

An examination of Table 129 shows that a 97.6 per cent yield of s was obtained from a 19.8 per cent concentration of sorbitol in practi 33 hr. under the conditions of the fermentation. The advantages o type of fermentation in comparison with the surface-growth typ apparent.

An active inoculum was prepared by growing *A. suboxydans* in a tion containing 100 g. of sorbitol, 5 g. of Difco yeast extract, 10 g. o cose, and 3.1 g. of calcium carbonat liter of solution. Calcium carbonate necessary component of this solution the gluconic acid formed from glu unless neutralized, will prevent sor from being produced. More uniform re were obtained from the inoculum using conditions of air flow, pressure, agitation that did not favor a quick dation of sorbitol.

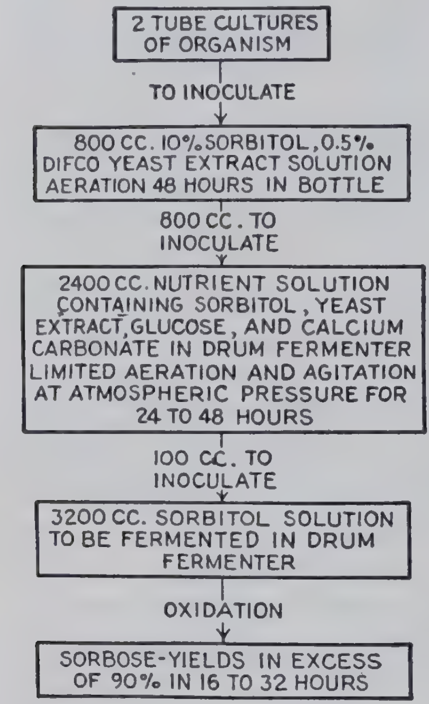

2 TUBE CULTURES OF ORGANISM
TO INOCULATE
800 CC. 10% SORBITOL, 0.5% DIFCO YEAST EXTRACT SOLUTION AERATION 48 HOURS IN BOTTLE
800 CC. TO INOCULATE
2400 CC. NUTRIENT SOLUTION CONTAINING SORBITOL, YEAST EXTRACT, GLUCOSE, AND CALCIUM CARBONATE IN DRUM FERMENTER LIMITED AERATION AND AGITATION AT ATMOSPHERIC PRESSURE FOR 24 TO 48 HOURS
100 CC. TO INOCULATE
3200 CC. SORBITOL SOLUTION TO BE FERMENTED IN DRUM FERMENTER
OXIDATION
SORBOSE-YIELDS IN EXCESS OF 90% IN 16 TO 32 HOURS

FIG. 79. Diagram of large-scale inoculum-preparation method for producing sorbose from sorbitol. [*Courtesy of P. A. Wells, J. J. Stubbs, L. B. Lockwood, and E. T. Roe, Ind. Eng. Chem.*, 29: 1385 (1937). *Copyright* 1937 *by the American Chemical Society and reprinted by permission of the copyright owner.*]

*Semi-plant-scale Production.* The p duction of L-sorbose from D-sorbitol *A. suboxydans* has been carried out s cessfully on a semi-plant scale substitu corn-steep liquor (3 g. per liter) for d yeast extract as the only source of nutri material (Wells et al., 1939). It was nec sary, however, to use octadecyl alcohol antifoam substance, to prevent excess frothing of the medium.

Control of the pH was accomplished adding to the nutrient medium an amo of calcium carbonate slightly in excess that required to neutralize the corn-st liquor and the gluconic acid produced fermentation from the small quantity glucose present in the sorbitol sirup used

Sorbitol, in concentrations as high as per cent, was oxidized to L-sorbose rapi and efficiently. It required a longer peri of time to oxidize the higher concentrations of D-sorbitol; for example, one case a 10 per cent sorbitol solution required only 13.5 hr. while 30 per cent solution required 45 hr. for the conversion of most of t sorbitol to sorbose.

Results of one large-scale experiment are given in Table 130.

*Use of Vats.* Vats are used in the commercial production of s bose.

*Maintenance of Stock Cultures.* Stock cultures or *A. suboxydans* m be carried on agar slants containing 5 per cent sorbitol or on malt extr agar slants.

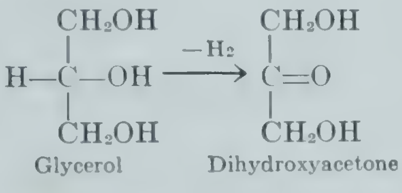
E 130. Typical Pilot-plant-scale Results on the Production of l-Sorbose from d-Sorbitol by *Acetobacter suboxydans* *

| | |
|---|---|
| Total sorbitol available (dry basis) | 163.4 kg. |
| Total sorbose produced | 151.0 kg. |
| Total sorbose recovered (3 crops) | 114.3 kg. |
| Weight yield based on sorbose produced | 92.4% |
| Weight yield based on sorbose recovered | 69.8% |

A. Wells, L. B. Lockwood, J. J. Stubbs, E. T. Roe, N. Porges, and E. A. k, *Ind. Eng. Chem.*, 31: 1518–1521 (1939). Copyright 1939 by the American al Society and reprinted by permission of the copyright owner.

## PRODUCTION OF DIHYDROXYACETONE FROM GLYCEROL

ydroxyacetone is an industrially important chemical compound l biologically through dehydrogenation, or oxidation, of glycerol tain acetic acid bacteria:



$$\mathrm{H{-}\overset{\overset{CH_2OH}{|}}{\underset{\underset{CH_2OH}{|}}{C}}{-}OH \xrightarrow{-H_2} \overset{\overset{CH_2OH}{|}}{\underset{\underset{CH_2OH}{|}}{C}}{=}O}$$

he preceding equation, oxygen is the hydrogen acceptor. Provided ey are sufficiently active, other hydrogen acceptors may be substi- for oxygen.

formation of dihydroxyacetone by microorganisms was first ed by Bertrand. His sorbose bacterium (*A. xylinum*) oxidized l to dihydroxyacetone. Since the original work by Bertrand, con- le research has been undertaken and several patents have been concerning the fermentation.

**anisms.** Various species of the genus *Acetobacter* may be used production of dihydroxyacetone from glycerol, but *A. suboxydans* *xylinum* are most frequently employed. *Bact. orleanense* and *i* have produced high yields of dihydroxyacetone. *Bact. xylinoides*, *onicum*, *A. dihydroxyacetonicum*, *A. melanogenus*, and *A. rancens* e form dihydroxyacetone from glycerol.

**mum Conditions for Fermentation.** A suitable supply of nutri- terial is necessary for a successful yield of dihydroxyacetone. Five it yeast water was used by Bertrand and some later investigators, has been replaced by the use of 0.5 per cent concentrations of yeast t. Underkofler and Fulmer (1937) obtained optimum yields of oxyacetone when using 0.10 to 0.30 per cent of potassium dihydro- osphate in addition to 0.5 per cent yeast extract. Malt extract and roducts have proved satisfactory as substitutes for yeast extract. tion, temperature, pH, and glycerol concentration are important . Visser't Hooft (1925) investigated the effect of aeration on l transformation and obtained the data shown in Table 131 in

TABLE 131. EFFECT OF AERATION ON THE OXIDATION OF GLYCEROL*

| Time | Glycerol transformed to dihydroxyacetone, per cent | |
|---|---|---|
| | With aeration | Without aeration |
| After 2 days | 35 | 11 |
| After 4 days | 84 | 30 |
| After 6 days | 90 | 57 |

* K. R. Butlin, "The Biochemical Activities of the Acetic Acid Bacteria," C
Research, Special Report 2, H.M. Stationery Office, London, 1936.

connection with the study he undertook involving *Acetobacte oxydans.*

Bernhauer and Schön found a medium 1 to 2 cm. deep to be op from the viewpoint of aeration.

In Table 132 are summarized some data concerning the optimum perature, pH, and glycerol concentration for the conversion of gl to dihydroxyacetone by certain specified acetic acid bacteria.

TABLE 132. SOME OPTIMUM CONDITIONS FOR THE OXIDATION OF GLYCEROL DIHYDROXYACETONE

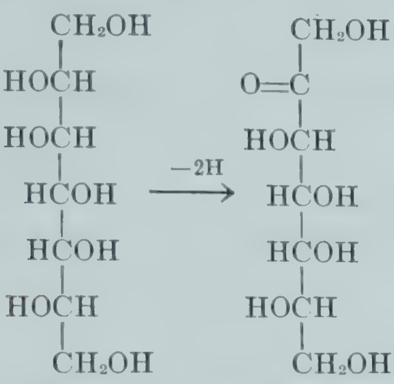
| Organism | Optimum temperature, °C. | Optimum pH | Optimum glycerol concentration, per cent | Investigato |
|---|---|---|---|---|
| Organism closely related to *Acetobacter xylinum* | 27–28 | 4–4.8 | 6–8 | Bernhauer a Schön (19 |
| *A. xylinum* | ..... | 5.5 | 2 | Cozic (1933) |
| *A. xylinum; A. dihydroxyacetonicum* | ..... | 5 | ... | Virtanen an Bärlund (1926) |
| *A. suboxydans* | 28–30 | 5.5–7.0 | 6 | Underkofler Fulmer (1937) |

Underkofler and Fulmer found that glycerol concentrations g than 6 per cent produced lower yields of dihydroxyacetone.

*Yields.* Yields of 85 to 95 per cent of dihydroxyacetone on the of the glycerol fermented are not uncommon.

## THE PERSEULOSE FERMENTATION

ulose is a rare ketoheptose. It may be prepared by the oxidation
drogenation of perseitol, an alcohol that occurs as a constituent
avocado (*Persea gratissima*):



$$\begin{array}{c} CH_2OH \\ | \\ HOCH \\ | \\ HOCH \\ | \\ HCOH \\ | \\ HCOH \\ | \\ HOCH \\ | \\ CH_2OH \end{array} \xrightarrow{-2H} \begin{array}{c} CH_2OH \\ | \\ O{=}C \\ | \\ HOCH \\ | \\ HCOH \\ | \\ HCOH \\ | \\ HOCH \\ | \\ CH_2OH \end{array}$$

Perseitol Perseulose

rand discovered perseulose in 1908, as a product of the oxidation
eitol by the sorbose bacterium, *Acetobacter xlyinum.*
n (1939) has carried out research, in cooperation with Wells,
. Lockwood, and Roe, concerning the production of perseulose from
ol, using *A. suboxydans*.
eneral, best results were obtained by Tilden when the medium
ed 0.3 per cent of neutralized corn-steep liquor (yeast extract, in
cent concentration, may be used in place of neutralized corn
0.3 per cent $KH_2PO_4$, 0.05 per cent glucose (promoted complete
on of perseitol), and 3 to 4 per cent of perseitol.
rotary drum fermentors, of the type described in Chap. 29, and
ons similar to those found most favorable for the production of
from sorbitol (the use of 3,200 ml. of culture per drum, an air flow
) ml. per min., a pressure of 30 lb., and a rotation speed of 13
3 to 4 per cent concentrations of perseitol were oxidized quanti-
to perseulose in 36 to 48 hr. at a temperature of 30°C.
later time, a pH of 6.4 was found to be optimum for the fermenta-
stead of the pH of 5.5, or less, which was used in the rotary drum
ors. Thus the time required for the conversion may have been
still further had the optimum pH been used.

## THE PRODUCTION OF GLUCONIC ACID

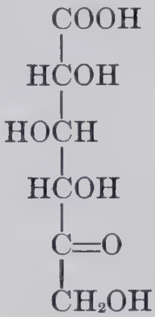
principal bacteria capable of producing gluconic acid in quantity
ucose belong to the group of acetic acid bacteria. Such acetic acid
as *Acetobacter oxydans* may be used. These bacteria are able,
avorable conditions, to oxidize glucose to gluconic acid in the
of an abundant supply of oxygen. Currie and Carter, in a patent
o them in 1933, suggested that glucose concentrations as high as
cent may be used, but 25 per cent is stated to be the optimum

concentration. From 0.2 to 2 per cent of mineral salts is added t mentation mash to satisfy the mineral requirements of the bact nutrient glucose solution is permitted to pass in a thin stre through an apparatus similar to a vinegar generator. The pres large amount of oxygen is thus assured. A temperature of 15 is advocated for the fermentation. Hermann, in an Austrian pat gested a similar plan for the production of gluconic acid.

Takahashi (1934) advocated the use of *Bacterium Hoshig* *rosea* and *Bact. industrium* var. *Hoshigaki* in the fermentation o or mannite solutions. Either soybeans or the extract of rice bran to the sugar solution to meet the nitrogen requirements of the fe tion organism. The fermentation is permited to run for 18 days 28°C. A very high yield is reported—as high as 103 per cent on t of the glucose.

## THE PRODUCTION OF 5-KETOGLUCONIC ACID

The structural formula of D-5-ketogluconic acid is as follows:



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{HCOH} \\ | \\ \mathrm{HOCH} \\ | \\ \mathrm{HCOH} \\ | \\ \mathrm{C{=}O} \\ | \\ \mathrm{CH_2OH} \end{array}$$

D-5-Ketogluconic acid

The literature concerning the formation of 5-ketogluconic ac been reviewed by Stubbs, Lockwood, Roe, Tabenkin, and Ward who described the production of this acid and 2-ketogluconic aci information which follows is based on their report.

**Organism Used.** A strain of *Acetobacter suboxydans* was use

**Preparation of Inoculum.** The inoculum was prepared in the ing manner: *A. suboxydans* was grown for about 2 days at 30°C. in of a sterile medium containing 5 per cent sorbitol and 0.5 per cen extract. This culture was employed to inoculate 200 ml. of a medium containing 2 per cent glucose and 0.5 per cent yeast e The medium was aerated with 200 ml. of air per min. while in glass gas-washing bottle (type 101a). After about 2 days' incuba 30°C., this culture was used to inoculate 3 liters of a sterile mediu taining 5 per cent glucose, 0.5 per cent yeast extract, and 37 g. of c carbonate in a 4-liter serological bottle. This medium was aerate 5 liters of air per minute. After about 2 days at 30°C., the inoculu ready for use in seeding the main production medium.

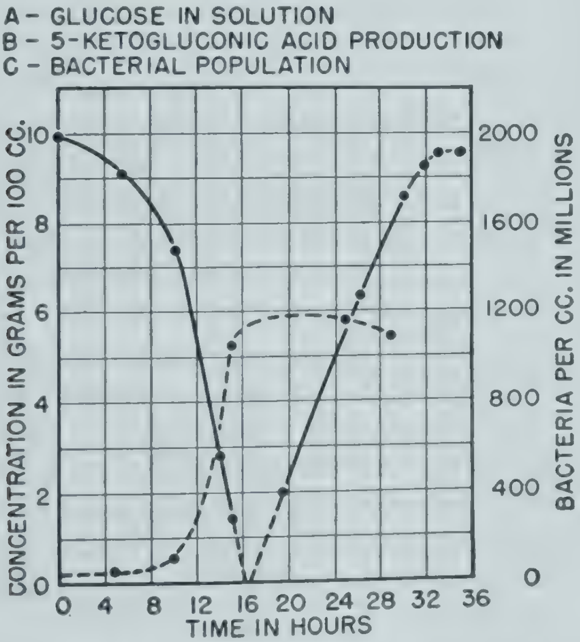
**Composition of Production Medium.** The production mediu prepared to contain the following components:

| Components | Amount |
|---|---|
| Commercial glucose | 118 g.* |
| Corn-steep liquor | 5 g. |
| Octadecyl alcohol † | 0.3 g. |
| Calcium carbonate ‡ | 27 g. |
| Distilled water to make | 1,000 ml. § |

unt to provide 10 per cent glucose concentration.
oam agent.
ized separately.
solution was sterilized by autoclaving at 20 lb. pressure for 20 min.

**itions of Production.** The conditions of fermentation were as : A charge of 3 liters of the production medium was placed in a lrum of the type described in Chap. 29. The drum was rotated p.m., while air was introduced at the rate of 1,600 ml. per min. red as exit gas at atmospheric pressure), and the pressure in the as maintained at 30 lb. per sq. in. The contents of the drum eded with 300 ml. of inoculum, prepared as described above. The ature was kept at 30°C.

**se of Fermentation.** In Fig. 80 is shown the course of a typical luconic acid fermentation.



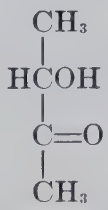
Course of typical 5-ketogluconic acid fermentation. [*Courtesy of J. J.* *L. B. Lockwood, E. T. Roe, B. Tabenkin, and G. E. Ward, Ind. Eng. Chem.,* (1940). *Copyright* 1940 *by the American Chemical Society and reprinted by* *n of the copyright owner.*]

s. Yields of approximately 90 per cent of 5-ketogluconic acid tained in 33 hr. from 10 per cent glucose solutions.

**ted Process.** A patented process for the production of 5-keto- acid has been described by Stubbs, Lockwood, Roe, and Ward A nutrient solution of glucose or calcium gluconate is acted upon by an active culture of *Acetobacter* under conditions of aeration a tation at 25 to 30°C. The nutrients include corn-steep liquor; urea acids, ammonium sulfate, or ammonium phosphate; potassium hy phosphate; and magnesium sulfate. Calcium carbonate, zinc car or other compounds are used as the neutralizing agents.

## THE PRODUCTION OF ACETYLMETHYLCARBINOL

Acetylmethylcarbinol, known also as acetoin, has the following tural formula:



$$\begin{array}{c} CH_3 \\ | \\ HCOH \\ | \\ C{=}O \\ | \\ CH_3 \end{array}$$

Acetylmethylcarbinol

Its production in high yields from 2,3-butanediol has been describ Fulmer, Underkofler, and Bantz (1943); Underkofler, Fulmer, Bant Kooi (1944); Sjolander and Eisenman (1946); and others.

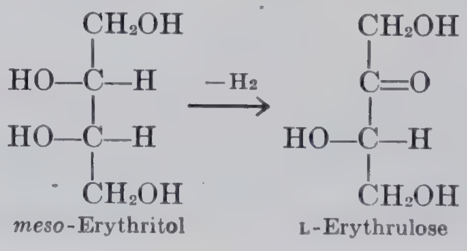
Fulmer and his associates found that yields amounting to 90 to 9 cent of the theoretical could be obtained from nutrient media conta the meso form of 2,3-butanediol. However, dextrorotatory 2,3-butan was not fermented. The following is an illustration of the proc employed by Fulmer and his coworkers: Two and one-half liters medium containing 10 per cent glycol, 0.5 per cent yeast extract, an per cent maltose and adjusted to an initial pH of 6 was sterilized 4-liter Erlenmeyer flask, cooled, and inoculated with 100 ml. of a 24 old culture of *Acetobacter suboxydans* (A.T.C.C. No. 621) grown butanediol–yeast-extract–maltose medium. The medium was aerated ing the conversion process.

Underkofler and his collaborators reported that only the meso levorotatory forms of 2,3-butanediol were oxidized to acetylmethy binol. Inasmuch as the 2,3-butanediol produced by *Aerobacter aero* is largely of the meso form, the 2,3-butanediol in the fermented li (beers) from such a fermentation may be converted largely to acetoi

Sjolander and Eisenman obtained 80 per cent conversion of the butanediol in beers that contained in excess of 6 per cent of the glyc

## THE PRODUCTION OF L-ERYTHRULOSE

L-Erythrulose is formed by the oxidation of *meso*-erythritol in ac ance with the following reaction:



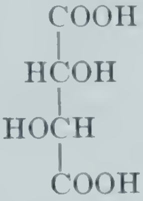
$$\begin{array}{c} CH_2OH \\ | \\ HO{-}C{-}H \\ | \\ HO{-}C{-}H \\ | \\ CH_2OH \end{array} \xrightarrow{-H_2} \begin{array}{c} CH_2OH \\ | \\ C{=}O \\ | \\ HO{-}C{-}H \\ | \\ CH_2OH \end{array}$$

*meso*-Erythritol L-Erythrulose

yields of L-erythrulose have been obtained by Whistler and ofler (1938), who employed the following production method: dium was prepared to contain 4.5 g. of *meso*-erythritol and 0.5 g. extract per 100 ml. The pH was 6.1. The medium was distributed nl. amounts in 2-liter Erlenmeyer flasks and sterilized for 20 min. ). steam pressure. The inoculum for a flask was 6 ml. of a 24-hr. of *Acetobacter suboxydans* (A.T.C.C. No. 621) grown in a medium lar composition to the one described above. The inoculated was incubated for 9 days at 28°C. About 95 per cent of the *meso*-ol was converted to L-erythrulose under the foregoing conditions.

## THE PRODUCTION OF D-TARTARIC ACID

acid, which has the following structural formula, may be pro- n whole or in part by fermentation methods.



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{HCOH} \\ | \\ \mathrm{HOCH} \\ | \\ \mathrm{COOH} \end{array}$$

D-Tartaric acid

let (1943) patented a process for producing D-tartaric acid from aqueous solutions of glucose using *Acetobacter suboxydans* .C. No. 621) and a catalyst.

xample of the process follows: *A. suboxydans* is grown for 3 days agar at 37°C. The culture is used to inoculate a liter of solution ng 10 per cent of glucose and 0.5 per cent yeast extract. This is for 48 hr. during incubation at 30°C. and used to seed a substrate ng 3 kg. of glucose, 1 liter of corn-steep liquor, and 30 liters of A vanadium compound is added in the proportion of 0.1 to 0.5 t, on the basis of the glucose, for the purpose of catalyzing the g of glucose to a 4-carbon compound.

onditions of fermentation were as follows: The pH is adjusted to 0 by the addition of a sterile aqueous solution of sodium carbonate. . pressure of 30 lb. per sq. in. is passed through the medium. The ture is maintained at an optimum of 28 to 30°C.

reaction is considered to be complete when there is no further in the pH after aeration for 30 min. The usual conversion time is hr.

tartaric acid is recovered as a crude potassium acid tartrate by the medium, adding a concentrated aqueous solution of potas- loride to the filtrate in such quantity that there will be 1 g. of m chloride for each 2 g. of D-tartaric acid, adjusting the pH to 3.5 etic acid, and filtering off the precipitate formed.

method patented by Pasternack and Brown (1940) and one d by Lockwood and Nelson (1951), D-5-ketogluconic acid pro- y fermentation or by chemical methods is converted to D-tartaric

acid through the action of an oxygen-containing gas in the prese
metallic catalysts.

In one illustration given by Pasternack and Brown, finely dis
oxygen is passed through 2 liters of an aqueous solution containing
of D-5-ketogluconic acid to which 10 g. of vanadium pentoxide was a
The reaction was carried out at 50°C. and was about complete in ap
mately 8 hr. The tartaric acid was recovered in the form of pota
acid tartrate. It is claimed by Pasternack and Brown that the acid
be prepared "substantially free of isomer" by their process.

## THE PRODUCTION OF FRUCTOSE

Fructose may be produced by two different principal methods by n
biological means. In one method, mannitol is converted to fructos
oxidation; in another, fructose accumulates as a by-product during
manufacture of dextran from sucrose.

**Fructose from Mannitol.** A method for the submerged culture
duction of D-fructose from D-mannitol by the use of *Acetobacter sub*
*dans* NRRL B-72 has been described by Peterson et al. (1956). Yiel
excess of 95 per cent fructose were obtained in shake flasks and in 30-l
50-gal., and 500-gal. fermentors.

Production in a 500-gal. fermentor was as follows: 300 gal. of prod
tion medium (18 per cent mannitol and 0.5 per cent dried brewers' y
No. 2019), adjusted to pH 5.8 with diluted $H_2SO_4$, was sterilized i
500-gal. fermentor for 30 min. at 122°C. After cooling to 28°C., it
inoculated with 30 gal. of a 21-hr. culture and maintained at 28°C. Fo
ing was controlled by adding 0.06 per cent nonsterile DC Antifoam
Emulsion in three equal batches (after sterilization and before inoc
tion, after 4 hr., and after 7 hr.). The aeration rate was 1.09 volume
air per volume of medium per minute; agitation was at 300 r.p.m. Yi
in excess of 95 per cent were obtained in 26 hr.

Data concerning inoculum development are presented in Table
Further details may be ascertained by reference to the original art
by Peterson and associates.

## KETOGENIC ACTIVITIES OF ACETOBACTER SPECIES IN GLUCOSE MEDIUM

Kulka and Walker (1954) studied the action of 14 species of *Acetob*
*ter* on glucose in a medium containing yeast water and calcium carbon
Each of the cultures produced a reducing acid (or acids) which yielde
soluble calcium salt (or salts). It was demonstrated that the redu
material consisted in part, or entirely, of calcium 2-keto-D-gluconat
the cases of 11 species. A number of species produced 5-ketogluconic
in addition to the 2-keto acid. Table 134 indicates the amounts of
acids formed by the cultures.

TABLE 133. DATA CONCERNING INOCULUM DEVELOPMENT *

| | Shaken-flask fermentations | 30-liter fermentations | 50-gal. fermentations | 500-gal. fermentations |
|---|---|---|---|---|
| Composition of medium used for inoculum.... | Mannitol, 7.0% ; Difco yeast extract, 0.5% ; $KH_2PO_4$, 0.1% ; pH to 5.8; † tap water | Mannitol, 7% ; Difco yeast extract, 0.5% ; $KH_2PO_4$, 0.1% ; pH to 5.8; † tap water | Mannitol, 18.0% ; brewers' yeast No. 2019, 0.5% ; $KH_2PO_4$, 0.1% ; pH to 5.8; † tap water | Mannitol, 18.0% ; brewers' yeast No. 2019, 0.5% ; $KH_2PO_4$, 0.1% ; pH to 5.8; † tap water |
| Volume of inoculum..... | 5% (second transfer) | 5% | 3 gal. | 30 gal. |
| Age of inoculum, hr. ... | 24 | 24 | 21 | 21 |
| Fermentor used for inoculum development ............... | Shake flask | Aerated bottle | 50-gal. fermentor | 50-gal. fermentor |
| Temperature, °C. ...... | 28 | 28 | 28 | 28 |

* M. H. Peterson et al., *Appl. Microbiol.,* **4**(6): 316 (1956).

† With dilute $H_2SO_4$.

Table 134. Production of 5-Ketogluconic Acid, 2-Ketogluconic Acid, an Reducing Acid by *Acetobacter* Species *

(Produced from glucose, 10 g., in presence of calcium carbonate, 3 g.

| Reference no. | Name | Glucose used, g. | Ca salt of 5-KGA † $\cdot 2\frac{1}{2}H_2O$, g. | Soluble Ca salts of reducing acids, g. glucose | Total ketonic and aldehydic acids (expressed as % of weight of glucose used) | R re p as ca so |
|---|---|---|---|---|---|---|
| A | B | C | D | E | F | |
| 1 | *A. gluconicum* N.C.I.B. 4739 | 9.87 | 5.25 | 2.13 | 76.5 | 3. |
| 2 | *A. turbidans* N.C.I.B. 6249, tested 1947 | 10.00 | .... | 4.49 | 75.0 | 4. |
| 3 | *A. turbidans* N.C.I.B. 6249, tested 1949 | 9.70 | 6.30 | 1.30 | 72.0 | |
| 4 | *A. suboxydans* N.C.I.B. 7069; A.T.C.C. 621 | 9.68 | 7.30 | 0.85 | 72.2 | 5.8 |
| 5 | *A. acetigenum* N.C.I.B 5346 | 9.12 | 2.60 | 2.6 | 69.5 ‡ | |
| 6 | *A. orleanense* (beer, 1946) | 9.61 | 1.70 | 1.67 | 42.5 | |
| 7 | *A. acetosum* (African vinegar, 1944) | 2.24 | .... | 0.43 | 32.0 ‡ | |
| 8 | *A. viscosum* N.C.I.B. 6426 | 9.36 | 2.50 | 0.45 | 28.5 | |
| 9 | *A. mobile* N.C.I.B 6428 | 9.99 | .... | 1.66 | 27.6 | 12.0 |
| 10 | *A. xylinum* N.C.I.B. 4112 | 9.33 | 0.60 | 1.25 | 27.2 ‡ | 3.8 |
| 11 | *A. acetigenum* (African vinegar, 1944) | 9.53 | 1.20 | 0.90 | 25.9 | 3.0 |
| 12 | *A. kützingianum* N.C.I.B. 3924 | 9.52 | 0.60 | 1.09 | 23.8 | 3.9 |
| 13 | *A. aceti* N.C.I.B. 6423 | 9.81 | .... | 1.11 | 18.8 ‡ | 4.1 |
| 14 | *A. ascendens* N.C.I.B. 4937 | 4.24 | .... | 0.47 | 18.5 ‡ | |
| 15 | *A. acidum-mucosum* N.C.I.B. 6429 | 3.07 | .... | 0.32 | 17.3 | |
| 16 | *A. acetosum* (beer, 1944) | 9.81 | .... | 1.00 | 17.0 ‡ | 3.2 |
| 17 | *A. acetosum* N.C.I.B. 6425 | 9.60 | .... | 0.81 | 14.0 | 4.6 |
| 18 | *A. capsulatum* N.C.I.B. 4943 | 9.72 | .... | 0.81 | 13.9 ‡ | 4.2 |
| 19 | *A. ascendens* (African vinegar, 1944) | 4.59 | .... | 0.34 | 12.3 | |
| 20 | *A. suboxydans* (beer, 1944) | 9.45 | 0.3 | 0.43 | 10.0 ‡ | |
| 21 | *A. acetosum* N.C.I.B. 2224 | 10.00 | .... | 0.58 | 9.7 | 4.8 |

* Courtesy of D. Kulka and T. K. Walker, *Arch. Biochem. and Biophys.*, 50 169 (1954).

† 5-KGA represents 5-ketogluconic acid.

‡ Presence of aldehydogluconic acid detected by naphthoresorcinol test. Cu nos. 1, 4, 8, 16, 17, 18, and 20 produced a little acetic acid. Culture nos. 8, 9, 1 15, 18, 19, 20, and 21 produced a little lactic acid.

## OXIDATIONS IN ACETOBACTER SUBOXYDANS

and Cheldelin (1952, 1953, 1954) and Hauge et al. (1954) have
out interesting research concerning oxidations in *Acetobacter*
*'ans* with the objective of obtaining information regarding the
ısms of its oxidative patterns. They showed (1952) that resting
4. *suboxydans* in a phosphate buffer could oxidize glycerol beyond
·droxyacetone stage and sorbitol beyond the sorbose stage. They
ıat repeated washing and centrifuging of the cells of this organism
·d most of the oxidizing activity with respect to glycerol and vir-
ll with respect to dihydroxyacetone. It was possible to restore
ivity by adding inorganic phosphate and diphosphopyridine-
de. The oxidation of glycerol beyond the dihydroxyacetone stage
ıbited completely by a $10^{-4}$ *M* concentration of 2,4-dinitrophenol.
r, this inhibitor appeared to have no effect on the oxidation of
of glycerol to dihydroxyacetone, or of sorbitol to sorbose.

s demonstrated (1953) that resting cells of *A. suboxydans* were
of oxidizing dihydroxyacetone, sorbose, lactic acid, pyruvic acid,
taldehyde but that these compounds, as well as alpha-glycerol
te, beta-glycerol phosphate, glucose-1-phosphate, glucose-6-phos-
nd fructose-1,6-diphosphate, and citraconic, mesaconic, itaconic,
-ketoglutaric, succinic, fumaric, malic, oxalacetic, acetic, and
acids were unable to support growth.

(1954) it was reported that cell-free extracts of *A. suboxydans*
le to bring about the transformation of ethanol to acetaldehyde,
to dihydroxyacetone, dihydroxyacetone to unknown products,
to unknown products, pyruvate to acetaldehyde, and acetalde-
acetate. The cell-free extracts, also intact resting cells, were not
lissimilate acetate or tricarboxylic acid cycle intermediates. A
urified ethanol dehydrogenase was prepared. For further details
interesting research, the reader is urged to consult the basic
ications.

### References on Acetic Acid and Vinegar

W.: "Industrial Fermentations." Reinhold Publishing Corporation, New
1926.
R. W., R. T. Wisthoff, and F. M. Hildebrandt: Packings for Vinegar Genera-
*Ind. Eng. Chem.*, **46**: 2023 (1954).
k, M. W.: Ueber die Arten der Essigbakterien, *Zentr. Bakt. Parasitenk.*, Abt.
: 209–216 (1898).
Manual of Determinative Bacteriology," 7th ed., The Williams & Wilkins
pany, Baltimore, 1957.
J.: *Jour. Chem. Soc.*, **49**: 172 (1886).
. E., P. Jowett, and T. K. Walker: Formation of D-Cellulose from Starch
Other Substrates by an *Acetobacter* Species, *Chemistry & Industry*, 1955,
7.
. V.: Vinegar from Waste Fruits, *Calif. Agr. Expt. Sta., Bull.* 287, 1917.
Commercial Fruit and Vegetable Products," 4th ed., McGraw-Hill Book
pany, Inc., New York, 1958.

——— and M. A. Joslyn: Home and Farm Preparation of Vinegar, *Calif. Ag* *Sta., Circ.* 332, 1934.

Fabian, F. W.: Honey Vinegar, *Mich. State Coll., Ext. Bull.* 149, 1935.

Fetzer, W. R.: Modern Practice in Vinegar Making, *Food Inds.,* **2**: 489–491

Foda, I. O., and R. H. Vaughn: The Nutritional Requirements of *Acet* *melanogenum* and Related Species, *Jour. Bact.,* **65**: 79–82 (1953).

——— and ———: Oxidation of Maltose by *Acetobacter melanogenum, Jour.* **65**: 233–237 (1953).

Frateur, J.: Essai sur la systematique des acetobacters, *Cellule,* **53** (fasc. 3): (1950).

Hall, A. N., I. Husain, K. S. Tiwari, and T. K. Walker: Nutritional Requirem *Acetobacter* Species: Inorganic Ammonium Salts as Sources of Nitrogen *Appl. Bact.,* **19**: 31–35 (1956).

———, C. Russell, and K. S. Tiwari: The Response of Certain *Acetobacter* to the Lactose Moiety of Pantothenic Acid, *Jour. Bact.,* **68**: 279–281 (1954)

———, G. A. Thomas, K. S. Tiwari, and T. K. Walker: Nutrition of *Aceto* Species, *Arch. Biochem.,* **46**: 485–487 (1953).

Hansen, A. E.: Making Vinegar by the Frings Process, *Food Inds.,* **7**: 277 (19

Hansen, E. C.: *Compt. rend. de Carlsberg,* **1**: 49 and 96 (1879).

Hassack, P.: *The Vinegar Bulletin,* New York, 1916–1918.

———: Vinegar Manufacture in the United States, *Chem. Age* (*N.Y.*), **29** (1921).

Hauge, J. G., T. E. King, and V. H. Cheldelin: Glycerol Oxidation in *Acetob* *suboxydans, Federation Proc.,* 13(1)(March, 1954).

Henneberg, W.: "Gärungsbakteriologie Praktikum," Parey, Berlin, 1909.

Hermann, S.: *Biochem. Zeit.,* **192**: 176 (1928).

———: *Biochem. Zeit.,* **205**: 297 (1929).

——— and P. Neuschul: *Biochem. Zeit.,* **233**: 129 (1931).

Hoyer, D. P.: Bijdrage tot de Kennis van de Azijnbacterien, dissertation, Leiden, Waltman, Delft, 1898.

Hromatkin, O., und H. Ebner: Untersuchungen über die Essiggärung. I. selgärung und Durchlüftungsverfahren, *Enzymologia,* **13** (fasc. 6): 36 (1949).

——— und ———: Untersuchungen über die Essiggärung. II. Sauerstoffmangel Fesselgärung, *Enzymologia,* **14** (fasc. 2): 96–106 (1950).

——— und ———: Untersuchungen über die Essiggärung. III. Über den Einflus Belüft auf die submerse Gärung, *Enzymologia,* **15** (fasc. 2): 57–69 (1951).

———, ———, und C. Czoklich: Untersuchungen über die Essiggärung. IV. Über Einfluss einer vollstandigen Unterbrechung der Belüftung auf die subn Gärung, *Enzymologia,* **15** (fasc. 3): 134–153 (1953).

———, G. Kastner, und H. Ebner: Untersuchungen über die Essiggärung. V. den Einfluss von Temperatur und Gesamtkonzentration auf die subm Gärung, *Enzymologia,* **15** (fasc. 6): 337–350 (1953).

Lafar: Handbuch der Technische Mykologie, 1914.

Leifson, E.: The Flagellation and Taxonomy of Species of *Acetobacter, Leeu* *hoek ned. Tijdskr.,* **20**: 102 (1954).

McCullough, E. C.: "Disinfection and Sterilization," 2d ed., Lea & Febiger, Phila phia, 1945.

Molinari, E.: Über die biochemische Dismutation nebst Untersuchungen über Essiggärung, *Biochem. Zeit.,* **216**: 187 (1929).

Mollenhauer, H. P.: Vinegar Manufacture Processes, Plants and Problems, *Manuf.,* 1956, pp. 47–51.

Neuberg, C., und F. Windisch: Über die Essiggärung und die chemischen Leistu der Essigbakterien, *Biochem. Zeit.,* **166**: 454 (1925).

Pasteur, L.: Études sur la Vinaigre, 1868.

Prescott, S. C., and B. E. Proctor: "Food Technology," McGraw-Hill Book pany, Inc., New York, 1937.

Rainbow, C., and G. W. Mitson: Nutritional Requirements of Acetic Acid Bact *Jour. Gen. Microbiol.,* **9**: 371–375 (1953).

I., R. Raghavendra, and J. L. Stokes: Nutrition of the Acetic Acid Bacteria, *ır. Bact.*, **65**: 405–412 (1953).

ɔll, J. L.: Brewing Bacteriology. IV. The Acetic Acid Bacteria, *Wallerstein bs. Communs.*, **11**(32): 27–39 (1948).

L. E.: Vinegar in Volume, *Food Eng.*, **25**(7): 88, 89, 135, 137, 139 (1953).

, F. W.: "The Microbiology of Foods," 2d ed., Garrard Press, Champaign, Ill., 14.

J., and T. K. Walker: *Acetobacter acidum-mucosum* Tosic & Walker, N. sp., Organism Forming a Starch-like Polysaccharide, *J. Gen. Microbiol.*, **4**(2): 2–195 (1950).

, T. K., and D. Kulka: Comparative Studies of *Acetobacter* Species with rticular Reference to Beer Contaminants, *Wallerstein Labs. Communs.*, **12**(36): 28 (1949).

and ———: Procedure for the Characterization of the Acetic Acid Bacteria, *ır. Soc. Chem. Ind., London,* **65**: 104 and 180 (1946).

and J. Tošić: The Characterization and Identification of Acetic Acid Bac- ia, *Jour. Inst. Brewing,* **52**(5): 238 (1946).

d, H., and A. Bertho: Über den Mechanismus des Oxydationsvorgänge. . Das Wesen der Essigsäure-Gärung, *Ann.,* **467**: 98 (1928).

, H. B., and T. K. Walker: Synthesis of Melibiose by an *Acetobacter* Species a Lactate-buffered Glucose Medium, *Chemistry & Industry,* 1955, p. 18.

## Some Patents

L. S.: Vinegar Generator System, U.S. Patent 2,156,428, May 2, 1939.

H.: Manufacture of Vinegar, U.S. Patent 1,880,381, Oct. 4, 1932.

n, J. J.: Process for Producing Vinegar, U.S. Patent 2,423,897, July 15, 1947.

V. E.: High Strength Cider Vinegar and Method of Making the Same, U.S. tent 2,153,849, Apr. 11, 1939.

## References on Production of Sorbose

uer, K., und B. Göhrlich: Oxydationen mittels Essigbakterien. V. Ver- ichende Versuche über die Darstellung von *l*-Sorbose mittels verschiedener kterien, *Biochem. Zeit.*, **280**: 375 (1935).

d, G.: Préparation biochimique du sorbose, *Compt. rend.*, **122**: 900 (1896).

Étude biochimique de la bactérie du sorbose, *Ann. chim. phys.*, (**8**)**3**: 180–288 04).

n, J., et J. L. Leefers: La préparation de sorbitol pur et de sorbose et luence de ce cétose sur le pourvoir conducteur de l'acide borique, *Rec. trav. n.*, **54**: 861 (1935).

E. I., J. W. Dunning, J. F. Guymon, and L. A. Underkofler: The Effect of Concentration of Sorbitol upon the Production of Sorbose by the Action of *tobacter suboxydans, Jour. Am. Chem. Soc.*, **53**: 1012 (1936).

, K., und B. Schiedt: Zur Darstellung der *l*-Sorbose, *Biochem. Zeit.*, **271**: 61 34).

Hooft, F.: Biochemische onderzoekingen over het geslacht *Acetobacter*, the- Eidgenössischen Technische Hoogeschool, Delft, 1925.

P. A., J. J. Stubbs, L. B. Lockwood, and E. T. Roe: Sorbose from Sorbitol. duction by Submerged Growths of *Acetobacter suboxydans, Ind. Eng. Chem.*, 1385 (1937).

L. B. Lockwood, J. J. Stubbs, E. T. Roe, N. Porges, and F. A. Gastrock, *Ind. ı. Chem.*, **31**: 1518–1521 (1939).

## References on Production of Dihydroxyacetone

ıer, K., und K. Schön: Oxydationen mittels *Bacterium xylinum*. Die Bildung Dioxyaceton aus Glycerin, *Hoppe-Seyler's Zeit. physiol. Chem.*, **177**: 107 28).

Bertrand, G.: Préparation biochimique du sorbose, *Bull. soc. chim.,* **(3)15**: 6 (1896).

———: Sur le produit d'oxydation de la glycérine par le bactérie du sorbose, *C rend.,* **126**: 842 (1898).

———: Préparation biochimique de la dioxyacetone cristallisée, *Compt. rend.,* 984 (1898).

———: Sur la préparation biochimique de la dioxyacetone (propanediolone), *soc. chim.,* **(3)19**: 502 (1898).

———: Étude biochimique de la bactérie, *Ann. chim. phys.,* **(8)3**: 181–288 (1

Butlin, K. R.: The biological production of dihydroxyacetone, *Jour. Soc. Chem.* **57**: 463 (1938).

Cozic, M.: Étude biochimique de *Bacterium xylinum,* thesis, André Lesot, Nem 1933.

Fischer, H. O. L., und H. Mildbrand: Über Dioxyaceton, *Ber.,* **57**: 707 (1924).

Kluyver, A. J., and F. J. G. de Leeuw: *Acetobacter syboxydans* een merkwaar azijnbacterie, *Tijdschr. Vergelijk. Geneesk.,* **10**: 170 (1924).

Neuberg, C., und E. Hoffman: Notiz über einfache Gewinnung von kristallisier Dioxyaceton auf biochemischem Wege, *Biochem. Zeit.,* **279**: 318 (1935).

Sazerac, R.: Sur une bactérie oxydante, son action sur l'alcool et la glycérine, *Co rend.,* **137**: 90 (1903).

Underkofler, L. A., and E. I. Fulmer: The Production of Dihydroxyacetone by Action of *Acetobacter suboxydans* upon Glycerol, *Jour. Am. Chem. Soc.,* **59**: (1937).

Van Niel, C. B.: Biochemistry of Bacteria, *Ann. Rev. Biochem.,* **6**: 595–620 (19

Virtanen, A. I., und B. Barlund: Die Oxydation des Glycerins zu Dioxyaceton du Bakterien, *Biochem. Zeit.,* **169**: 169 (1926).

——— and M. Nordlund: LXIV. An Improved Method for the Preparation of Di droxyacetone, *Biochem. Jour.,* **27**: 442 (1933).

### Some Patents

British Patent 269,950, 1926.

Currie, J. N., and A. Finlay: Process of Fermentation, U.S. Patent 1,908,225, 1933.

Dutch Patent 27,804, 1932.

German Patent 563758, 1930.

Kamlet, J.: Production of *d*-Tartaric Acid by Fermentation, U.S. Patent 2,314,8 Mar. 23, 1943.

Lockwood, L. B., and E. N. Nelson: Preparation of D-Tartaric Acid, U.S. Pate 2,559,650, 1951.

Pasternack, R., and E. V. Brown: Preparation of D-Tartaric Acid, U.S. Pate 2,197,021, Apr. 16, 1940.

Sjolander, N., and W. Eisenman: Process for Production of 3-Hydroxy-2-Butano U.S. Patent 2,401,778, June 11, 1946.

Stubbs, J. J., L. B. Lockwood, E. T. Roe, and G. E. Ward: Fermentation Proc for the Production of 5-Ketogluconic Acid, U.S. Patent 2,318,641, May 11, 19

### General References on Biochemical Activities of Acetic Acid Bacteria

Bernhauer, K.: Biochemie der Essigbakterien, *Ergeb. Enzymforsch.,* **7**: 246–2 (1938).

———: "Gärungschemisches Praktikum," 2d ed., Springer-Verlag, Berlin, Vienn 1939.

——— und B. Görlich: Oxydationen mittels Essigbakterien. IV. Über die Bildu von 2-Ketogluconsäure durch *Bact. gluconicum, Biochem. Zeit.,* **280**: 367 (193

Butlin, K. R.: "The Biochemical Activities of the Acetic Acid Bacteria," Chemist Research, Special Report 2, H.M. Stationery Office, London, 1936.

———: Aerobic Breakdown of Glucose by *Bact. suboxydans, Biochem. Jour.,* 3 1870 (1936).

LXVII. The Enzyme System of *Bact. Suboxydans.* I. Variation of Aerobic
ivity with Age of Culture, *Biochem. Jour.*, **32**: 508 (1938).
g. J. W., E. I. Fulmer, and L. A. Underkofler: The Oxidation of *i*-Inositol
the Action of *Acetobacter suboxydans, Iowa State Coll. Jour. Sci.*, **15**(1):
46 (1940).
, E. I., A. C. Bantz, and L. A. Underkofler: The Use of Alfalfa Extract to
ply Nutrients for the Growth and Chemical Activities of *Acetobacter sub-*
*dans, Iowa State Coll. Jour. Sci.*, **18**(4): 369–376 (1944).
nd L. A. Underkofler: Oxidation of Polyhydric Alcohols by *Acetobacter sub-*
*dans, Iowa State Coll. Jour. Sci.*, **21**(3): 251–270 (1947).
———, and A. C. Bantz: The Production of Acetylmethylcarbinol by the
ion of *Acetobacter suboxydans* upon 2,3-Butylene Glycol, *Jour. Am. Chem.*
., **65**: 1424–1427 (1943).
D. E., and H. Beinert: Biological Oxidations, *Ann. Rev. Biochem.*, **24**: 1–44
55).
. N., D. Kulka, and T. K. Walker: Formation of Arabinose, Ribulose and
tronic Acid from 2-Keto-D-gluconic Acid, *Biochem. Jour.*, **60**: 271–274 (1955).
R. M., E. B. Tilden, and C. S. Hudson: The Oxidation of Sugar Alcohols by
*tobacter suboxydans, Jour. Am. Chem. Soc.*, **60**: 1201 (1938).
n, S., und P. Neuschul: Zur Biochemie der Essigbakterien, zugleich ein
schlag für eine neue Systematik, *Biochem. Zeit.*, **223**: 129 (1931).
. E., and V. H. Cheldelin: Oxidative Dissimilation in *Acetobacter suboxydans,*
sphorylative and Non-Phosphorylative, *Jour. Biol. Chem.*, **198**: 127–141
52).
nd ———: Sources of Energy and the Dinitrophenol Effect in the Growth of
*tobacter suboxydans, Jour. Bact.*, **66**: 581–584 (1953).
nd ———: Oxidations in *Acetobacter suboxydans, Biochim. et Biophys. Acta,*
108–116 (1954).
, A. J., and A. G. J. Boezaardt: On the Oxidation of Glucose by *Acetobacter*
*oxydans, Rec. trav. chim.*, **57**: 609 (1938).
D., and T. K. Walker: The Ketogenic Activities of *Acetobacter* Species in a
cose Medium, *Arch. Biochem.*, **50**: 169–179 (1954).
F.: I constituenti degli aceti di fermentazione atti a fissare iodio in soluzione
lina, *Chimica e industria (Milan)*, **35**: 70 (February, 1953).
, M. H., W. C. Friedland, F. W. Denison, Jr., and J. C. Sylvester: The Con-
ion of Fructose by *Acetobacter suboxydans, Appl. Microbiol.*, **4**: 316–322
6).
k, T.: VII. Sur la configuration de la meso-inosite, de la scylitte et d'un
se obtenu par voie biochimique (scyllo-ms-inosose), *Helv. Chim. Acta*, **25**:
752 (1942).
J. J., L. B. Lockwood, E. T. Roe, B. Tabenkin, and G. E. Ward: Bacterial
luction of Ketogluconic Acids from Glucose, *Ind. Eng. Chem.*, **32**: 1626
0).
E. B.: The Preparation of Perseulose by Oxidation of Perseitol with *Aceto-*
*er suboxydans, Jour. Bact.*, **37**: 629–637 (1939).
fler, L. A., A. C. Bantz, and W. H. Peterson: Growth Factors for Bacteria.
. Growth Requirements of *Acetobacter suboxydans, Jour. Bact.*, **45**(2): 183–
(1943).
E. I. Fulmer, A. C. Bantz, and E. R. Kooi: The Fermentation of the Stereo-
eric 2,3-Butanediols by *Acetobacter suboxydans, Iowa State Coll. Jour. Sci.,*
): 377–379 (1944).
R. H.: The Acetic Acid Bacteria, *Wallerstein Labs. Communs.*, **5**(14): 5–26
2).
nn, H. J.: Zur Physiologie der Essigbakterien. *Zentr. Bakt. Parasitenk.*,
II, **38**: 451 (1913).
. R. L., and L. A. Underkofler: The Production of *l*-Erythrulose by the
n of *Acetobacter suboxydans* upon Erythritol, *Jour. Am. Chem. Soc.*, **60**:
-2508 (1938).

## CHAPTER 24

# THE PROPIONIC ACID FERMENTATION

**Historical.** In 1841 Nöllner isolated a "Pseudo-Essigsäure" (pse acetic acid) from the decomposition products of tartaric acid. This stance is believed to have been largely propionic acid (CH$_3$CH$_2$CO

Fitz, in a series of articles on fermentation (1876–1884), discusse morphology and biochemical activities of the bacteria producing pr onic acid from calcium malate and from calcium lactate.

Orla-Jensen (1898) carried out research concerning the formatio eyes in Emmenthaler cheese. Later von Freudenreich and Orla-Jen (1898) reported the first description of the isolation of microorgani causing the propionic acid fermentation. They studied the propionic a bacteria rather extensively, especially in relation to cheese.

As the result of investigations carried out to discover the reasons w Emmenthaler cheese manufactured in the United States was lacking characteristic flavor and why the eye formation was deficient or abn mal, Sherman and Shaw (1923) supplied much important information respect to the propionic acid bacteria; for example, they demonstrated relationship between the propionic acid bacteria and the production o high-grade Emmenthaler cheese. Sherman (1924) described the use pure cultures of propionic acid bacteria, *Bacterium acidi propionici d*, ensure the production of Emmenthaler cheese with characteristic fla and with normal eye development. This organism did not suppress growth of undesirable bacteria, however. Cultures of *Lactobacillus b garicus* have long been used in cheese making to inhibit the developme of the wrong types of bacteria.

Whittier and Sherman (1923) studied the factors affecting the propio acid fermentation and, with Albus, the rates of fermentation of lacto galactose, glucose, sucrose, and maltose (Whittier et al., 1924).

Virtanen (1923, 1925) has studied the mechanism of the propionic a fermentation.

Van Niel's dissertation on the propionic acid bacteria was publish in 1928. This comprehensive monograph will prove to be of much val to anyone interested in the propionic acid bacteria.

Werkman, Wood, Stone, and their associates have carried out co siderable research on the propionic acid fermentation, particularly respect to the mechanism of the fermentation. Some of their work w be discussed later.

Tatum, Peterson, and their coworkers (1936*a*, *b*, *c*) have studied t effect of growth factors on propionic acid bacteria.

**Factors Affecting the Fermentation.** *The Organism.* Propionic ac ia, in general, may be characterized as Gram-positive, catalase e, nonsporeforming, nonmotile, facultative aerobes.

Niel lists eight main species (with their synonyms) on the basis phological, cultural, and biochemical differences:

*onibacterium freudenreichii* (*Bacterium acidi propionici a* von Freudenreich Orla-Jensen, etc.)
*senii* (*Bact. acidi propionici b* von Freudenreich and Orla-Jensen)
*terssonii* (*Bact. acidi propionici c* Troili Petersson)
*rmanii* (*Bact. acidi propionici d* Sherman)
*tosaceum* (*Bacillus acidi propionici* von Freudenreich and Orla-Jensen)
*rum* (*Bact. acidi propionici* var. *rubrum.* Thoeni and Allemann)
*enii* (*Bact. acidi propionici* var. *rubrum.* Thoeni and Allemann)
*hnicum*

ldition to these, the following are described in "Bergey's Manual of ninative Bacteriology" (1957):

*finosaceum* Werkman and Kendall (1931)
*binosum* Hitchner (1934)
*e* Hitchner (1934)

*chnicum* has the ability to ferment starch, dextrin, and glycogen— previously described propionic acid bacteria did not possess this . *P. thoenii* produces propionic acid and acetic acid from a glucose n containing yeast extract in a molecular ratio of approximately cording to Van Niel.

pionic acid bacteria may be isolated from a number of sources: heeses, and other dairy products; silage; soil; the excreta of cattle; her sources.

*Carbon Source.* A large number of raw materials have been utis sources of carbon by different species of *Propionibacterium.* naterials fermented include lactose, sucrose, maltose, glucose, raffi- rabinose, xylose, glycogen, dextrin, and starch; lactic, tartaric, inic acids; glycerol and mannitol; and proteins, protein deriva- nd fats. Lactose and low-priced carbohydrates would undoubt- used in the industrial production of propionic acid.

*Nitrogen Source.* Propionic acid bacteria may utilize several n-containing compounds. One of the most satisfactory nitrogen is yeast extract, at a concentration of approximately 0.4 per cent. es, whey, and corn meal may be utilized, especially in the associa- other bacteria, such as *Proteus mirabilis, Streptococcus lactis,* or *i.*

man suggests that the increased production of propionic acid nt from the association of bacteria may be due to the fact that acid is more readily utilized than lactose by the propionic acid a. Van Niel suggests that the stimulating effect may be due to an ion in the nitrogenous compounds of the medium.

m and his associates state that the propionic acid bacteria may to utilize ammonia as the only source of nitrogen when suitable tory substances are present. They suggest that yeast extract er complex nitrogen sources may be effective because they contain rogenous growth factors in addition to available nitrogen.

The nitrogen source is of much importance for it influences t and the completeness of fermentation and also the ratio of propion to acetic acid.

*Growth Factors.* It has been stated repeatedly in some of the literature that a complex source of nitrogen was essential for the of propionic acid bacteria. Recent research has indicated that factors rather than complex nitrogen sources are most important.

According to Wood and his associates (1936), amino acids are ben but not essential for the growth of propionic acid bacteria.

Wood and his coworkers (1937) obtained an ether-soluble facto yeast extract, which was indispensable for the growth of all the cultu propionic acid bacteria tested on a synthetic medium that cont ammonium sulfate as the nitrogen source. This factor has been in potato extract, corn extract, corn steep, and liver extract, in ad to yeast extract. It is a nonvolatile acid, soluble in chloroform, ben ether, and xylene but almost insoluble in petroleum ether. It ma adsorbed on Norit and eluted with acid-alcohol. The factor is replaced by a mixture containing vitamin $B_1$, nicotinic acid, pimelic uracil, beta-alanine, and pantothenic acid.

Vitamin $B_1$ (thiamin) stimulated the growth of propionic acid bac especially in the presence of amino acids. But not all propionic bacteria require vitamin $B_1$ for vigorous growth, according to Ta and his associates (1936).

Riboflavin (Wood et al., 1937), in a concentration of 0.05 $\gamma$ per stimulated the growth of propionic acid bacteria in a medium contai ammonium sulfate.

For a further discussion of this subject the reader is urged to con the various papers on growth factors that are cited at the end of chapter.

*The pH.* Most favorable results are obtained when the pH is adju to 6.8 to 7.2, a pH of 7 being usually preferred.

*The Temperature.* The optimum temperature for fermentatio approximately 30°C.

*Duration of Fermentation.* The fermentation normally requires 12 days, but, by adapting the fermentation to a semicontinuous b Van Niel demonstrated that the fermentation time may be considera reduced.

**Composition of Some Culture Media.** Van Niel used a cul medium containing yeast extract and 2 per cent sodium lactate, adju to a pH of 7 in some of his researches. Another medium used by contained yeast extract, 2 per cent glucose, and 2 per cent calc carbonate.

Tatum, Peterson, and Fred have cultured the organisms on a med containing 1 per cent malt sprouts, 1 per cent glucose, and calc carbonate.

A medium consisting of 5 g. of lactose, 5 g. of calcium carbonate, 1 g. of dried yeast in 100 ml. of water, with the pH adjusted to appr mately 7, was used by Whittier and Sherman under certain conditions asal medium containing 1 per cent glucose, 0.6 per cent sodium ·. 0.3 per cent ammonium sulfate, and Speakman's inorganic salts in ncentration (0.25 g. $K_2HPO_4$, 0.25 g. $KH_2PO_4$, 0.1 g. $MgSO_4 \cdot 7H_2O$, ;. NaCl, 0.005 g. $FeSO_4 \cdot 7H_2O$, 0.005 g. $MnSO_4 \cdot 4H_2O$, and 1,000 ml. was used by Wood and his coworkers (1938) in some of their h.

**ducts of the Fermentation.** The main end products of the pro- acid fermentation are propionic acid, acetic acid, and carbon diox- mall amounts of succinic acid are frequently produced, while nethylcarbinol is occasionally formed by certain species.

**o of Propionic Acid to Acetic Acid.** The ratio of propionic to acid varies according to the species, the nitrogen source, and other . In a medium containing glucose and yeast extract, the acids were ed in a ratio of approximately 5:1 by *P. thoenii.* Under similar ons the acids were formed in a ratio of about 3:1 by *P. rubrum.* using *P. shermanii* (*Bact. acidi propionici d*), Whittier and Sher- oserved a fairly constant ratio of 2 molecules of propionic acid to cule of acetic acid.

**ds.** Usually more than 75 per cent of the fermented sugar may ounted for as propionic and acetic acids, while less than 20 per cent for carbon dioxide production.

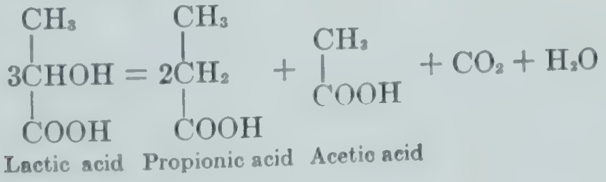
ne experiment, in which the medium contained yeast extract, 2 per lucose, and 2 per cent calcium carbonate, Van Niel obtained the ng results: 13.24 g. glucose fermented; and 8.62 g. propionic acid, acetic acid, 1.363 g. carbon dioxide, and 0.45 g. succinic acid ed.

ttier and Sherman have determined the conditions whereby yields roximately 2.4 lb. of propionic acid and 1 lb. of acetic acid may ained from 5 lb. of lactose after a fermentation period of 12 days ., using a mixed culture of *P. shermanii* and *L. casei* as the inocu- ields of 85 per cent or greater usually required a fermentation of 2 weeks or longer.

. The propionic acid bacteria determine the taste and flavor as the characteristic eye development in Emmenthaler cheese. ionic acid is used in the manufacture of perfumes. Ethyl propio- a solvent for pyroxylin. The mixture of propionic and acetic acids distilled to produce a mixture of acetone, methylethyl ketone, thyl ketone, substances which are solvents.

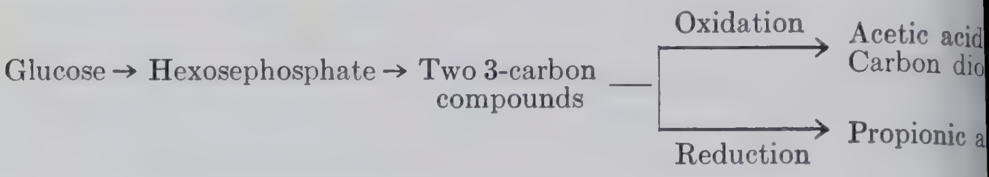
e quantities of propionic acid could be manufactured industrially nentation, provided that a demand arose for the acid.

**Mechanism of the Propionic Acid Fermentation.** Fitz (1878) d that lactic acid was converted by propionic acid bacteria in ance with the following equation:

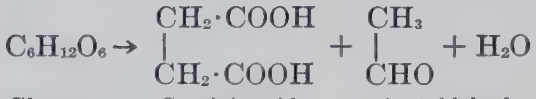


$$3\,CH_3{-}CHOH{-}COOH = 2\,CH_3{-}CH_2{-}COOH + CH_3{-}COOH + CO_2 + H_2O$$

Virtanen, Virtanen and Karström (1931), and Van Niel were opinion that two 3-carbon molecules were formed from glucose after phorylation, one of these being oxidized to acetic acid and carbon di while two other molecules were being reduced to propionic acid.


Glucose → Hexosephosphate → Two 3-carbon compounds
Oxidation
Acetic acid
Carbon dio
Reduction
Propionic a

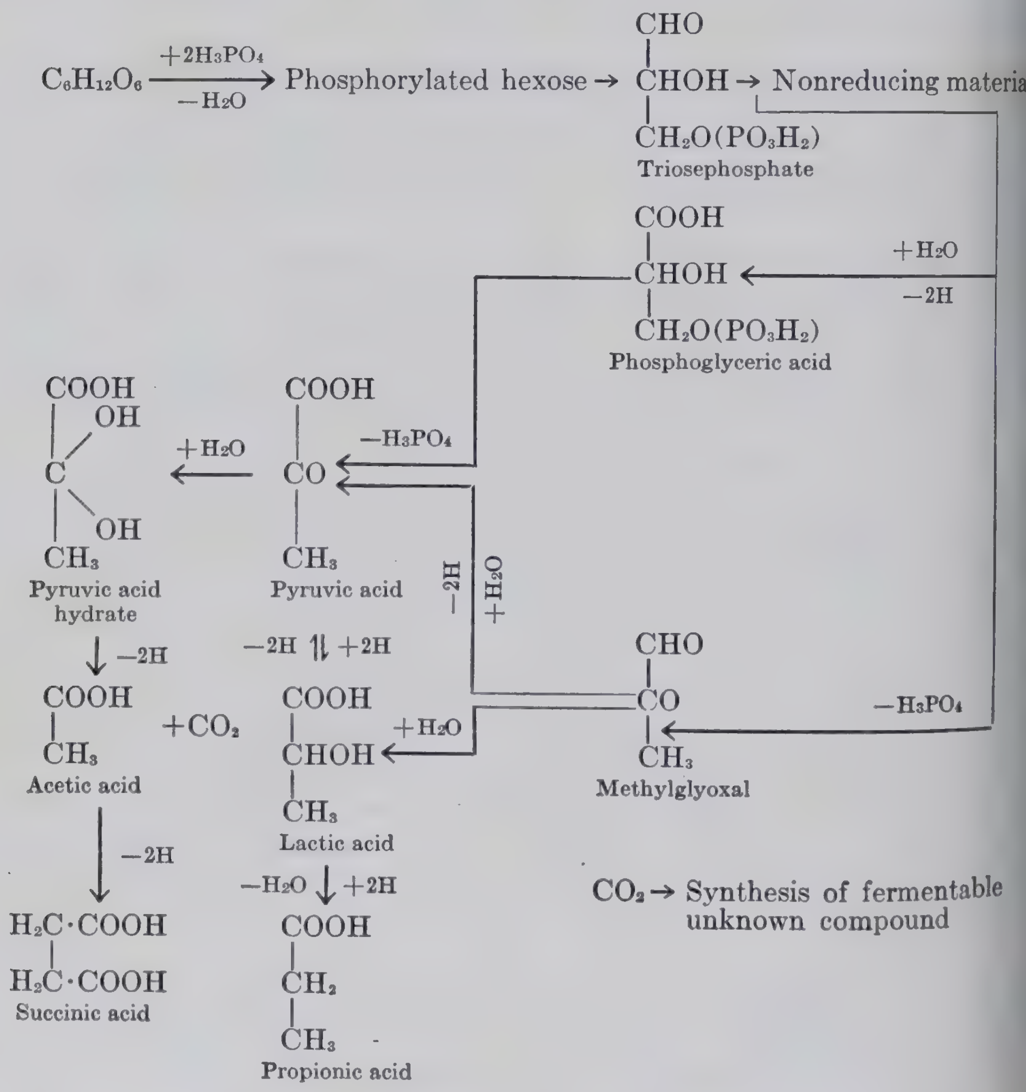
Van Niel proposed that pyruvic acid acted as an intermediate. V nen assumed that succinic acid arose in the following manner glucose:



$$C_6H_{12}O_6 \rightarrow \begin{matrix} CH_2\cdot COOH \\ | \\ CH_2\cdot COOH \end{matrix} + \begin{matrix} CH_3 \\ | \\ CHO \end{matrix} + H_2O$$

Glucose Succinic acid Acetaldehyde

Wood, Stone, and Werkman (1937) have proposed the following sch for the dissimilation of glucose by propionic acid bacteria:


C6H12O6
+2H3PO4
−H2O
Phosphorylated hexose →
CHO
CHOH
CH2O(PO3H2)
→ Nonreducing material
Triosephosphate
COOH
CHOH
CH2O(PO3H2)
+H2O
−2H
Phosphoglyceric acid
COOH
OH
C
OH
CH3
Pyruvic acid hydrate
+H2O
COOH
CO
CH3
Pyruvic acid
−H3PO4
−2H
+H2O
−2H
+2H
−2H
COOH
CH3
Acetic acid
+CO2
COOH
CHOH
CH3
Lactic acid
+H2O
CHO
CO
CH3
Methylglyoxal
−H3PO4
−2H
H2C·COOH
H2C·COOH
Succinic acid
−H2O
+2H
COOH
CH2
CH3
Propionic acid
CO2 → Synthesis of fermentable unknown compound

viche (1948) suggested that propionic acid was formed from pyru-
d by 6 major reactions which follow:

$$H_3COCOOH + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H$$

$$I_3COCOOH + CO_2 \rightarrow HOOCCH_2COCOOH$$

$$)OCCH_2COCOOH + 2H \rightarrow HOOCCH_2CHOHCOOH$$

$$)OCCH_2CHOHCOOH \rightarrow HOOCCH = CHCOOH + H_2O$$

$$)OCCH = CHCOOH + 2H \rightarrow HOOCCH_2CH_2COOH$$

$$)OCCH_2CH_2COOH \rightarrow CH_3CH_2COOH + CO_2$$

over-all equation, which follows, seems to account for the end prod-
rmed reasonably well:

$$3CH_3COCOOH + H_2O \rightarrow CH_3CH_2COOH + 2CH_3COOH + 2CO_2$$

er, it is believed that carbon dioxide fixation occurs by way of
acid rather than by way of oxalacetic acid.

reader is referred to the discussions of Clifton (1957) and of
nn (1955) for further information on this subject.

## Some References concerning the Propionic Acid Fermentation

C. E.: "Introduction to Bacterial Physiology," McGraw-Hill Book Company,
., New York, 1957.

ie, E. A.: Mechanism of Propionic Acid Fermentation by *Propionibacterium*
*tosaceum*, *Jour. Bact.*, **56**: 811–820 (1948).

, H. G. Wood, and C. H. Werkman: The Aerobic Dissimilation of Lactic
d by the Propionic Acid Bacteria, *Jour. Bact.*, **31**: 595 (1936).

: Über die Gährung des Glycerins, *Ber.*, **9**: 1348 (1876).

Über Schizomyceten—Gährungen. II. Glycerin, Mannit, Stärke, Dextrin,
., **10**: 276 (1877).

Über Schizomyceten—Gährungen III, *Ber.*, **11**: 42 (1878).

Über Spaltpilzgährungen, *Ber.*, **11**: 1890 (1878); **12**: 474 (1879); **13**: 1309
0).

II. Über Doppelsalze der niedrigen Fettsäuren, *Ber.*, **14**: 1084 (1881).

Über Spaltpilzgährungen, *Ber.*, **15**: 867 (1882); **16**: 844 (1883); **17**: 1188
4).

M., E. B. Fred, and W. H. Peterson: The Fermentation of Pentoses by Cer-
Propionic Acid Bacteria, *Zentr. Bakt. Parasitenk.*, Abt. II, **82**: 379 (1930).

reich, E. von, und S. Orla-Jensen: Über die Emmentalerkäse stattfindende
pionsäuregärung, *Zentr. Bakt. Parasitenk.*, Abt. II, **17**: 529 (1906).

ot, C., und E. L. Tatum: Über einen Aktivator des Stoffwechsels der Pro-
nsäurebakterien, *Biochem. Zeit.*, **267**: 360 (1933).

r, E. R.: Some Physiological Characteristics of the Propionic-acid Bacteria,
*r. Bact.*, **28**: 473 (1934).

S. A., and F. Saunders: Accessory Growth Factors for Bacteria and Related
roorganisms, *Bact. Revs.*, **2**: 117 (1938).

. G., R. Ross, and K. C. Blanchard: Is Vitamin $B_2$ the Accelerating Factor
he Fermentation of Sugar by Propionic Acid Organisms, *Philippine Jour. Sci.*,
493 (1936).

. E., and H. T. Herrick: Production of Organic Acids from Carbohydrates
Fermentation, *U.S. Dept. Agr., Circ.* 216, May, 1932.

sen, S.: Studien über die Lochbildung in den emmanthaler Käsen, *Zentr.*
*t. Parasitenk.*, Abt. II, **4**: 217, 265, 325 (1898).

———: "Dairy Bacteriology," McGraw-Hill Book Company, Inc., Blakiston I New York, 1931.

Pett, L. B., and A. M. Wynne: The Metabolism of Propionic Acid Bacteria. Degradation of Phosphoric Acid Esters by *Propionibacterium Jensenii* (va *Trans. Roy. Soc. Can.*, Sec. V, **27**: 119 (1933).

Shaw, R. H., and J. M. Sherman: The Production of Volatile Fatty Acids and Dioxide by Propionic Acid Bacteria with Special Reference to Their A Cheese, *Jour. Dairy Sci.*, **6**: 303 (1923).

Sherman, J. M.: The Cause of Eyes and Characteristic Flavor in Emmenthal Cheeses, *Jour. Bact.*, **6**: 379 (1921).

———: The Use of Bacterial Cultures for Controlling the Fermentation in E thal Cheese, *Proc. World's Dairy Congr.*, **1**: 287 (1923).

——— and R. H. Shaw: Associative Bacterial Action in the Propionic Acid Fer tion, *Jour. Gen. Physiol.*, **3**: 657 (1921).

——— and ———: The Propionic Fermentation of Lactose, *Jour. Biol. Che* 695 (1923).

Snell, E. E., F. M. Strong, and W. H. Peterson: Growth Factors for Ba VIII. Pantothenic and Nicotinic Acids as Essential Growth Factors for and Propionic Acid Bacteria, *Jour. Bact.*, **38**: 293 (1939).

Stone, R. W., H. G. Wood, and C. H. Werkman: Activities of the Lower Fatty by Propionic Acid Bacteria, *Jour. Bact.*, **30**: 652 (1935).

———, ———, and ———: Activation of the Lower Fatty Acids by Propionic Bacteria, *Biochem. Jour.*, **30**: 624 (1936).

Tatum, E. L., W. H. Peterson, and E. B. Fred: Essential Growth Factors for pionic Acid Bacteria. I. Sources and Fractionation, *Jour. Bact.*, **32**: 157 (1

———, H. G. Wood, and W. H. Peterson: Essential Growth Factors for Pro Acid Bacteria. II. Nature of the Neuberg Precipitate Fraction of Potato placement by Ammonium Sulphate or Certain Amino Acids, *Jour. Bact.* 167 (1936*b*).

———, ———, and ———: Growth Factors for Bacteria. V. Vitamin $B_1$, a Gr Stimulant for Propionic Acid Bacteria, *Biochem. Jour.*, **30**: 1898 (1936*b*).

Thimann, K. V.: "The Life of Bacteria," The Macmillan Company, New 1955.

Van Niel, C. B.: The Propionic Acid Bacteria, thesis, Eidgenössischen Techn Hoogeschool, Delft, September, 1928.

Virtanen, A. I.: Propionic Acid Fermentation, *Soc. Sci. Fennica, Commentat Phys.-Math.*, **1**(36): 1 (1923); **2**(20): 1 (1925).

——— and H. Karström: *Acta Chem. Fennica*, Ser. B, **7**: 17 (1931).

Werkman, C. H., and R. W. Brown: The Propionic Acid Bacteria. II. Classifica *Jour. Bact.*, **26**: 393 (1933).

———, R. M. Hixon, E. I. Fulmer, and C. H. Rayburn: The Production of pionic Acid from Pentoses by *Propionibacterium pentosaceum*, *Proc. Iowa Sci.*, **36**: 111 (1929).

——— and S. E. Kendall, *Iowa State Coll. Jour. Sci.*, **6**: 17 (1931).

Whittier, E. O., and J. M. Sherman: Propionic Acid and Ketones from Whey, *Eng. Chem.*, **15**: 729 (1923).

———, ———, and W. R. Albus: The Rates of Fermentation of Sugars by the pionic Organisms, *Ind. Eng. Chem.*, **16**: 122 (1924).

Wood, H. G., A. A. Andersen, and C. H. Werkman: Growth Factors for Propi and Lactic Acid Bacteria, *Proc. Soc. Exptl. Biol. Med.*, **36**: 217 (1937).

———, ———, and ———: Nutrition of the Propionic Acid Bacteria, *Jour. B* **36**: 201 (1938).

———, R. W. Stone, and C. H. Werkman: The Intermediate Metabolism of Propionic Acid Bacteria, *Biochem. Jour.*, **31**: 349 (1937).

———, E. L. Tatum, and W. H. Peterson: Growth Factors for Bacteria. IV. An A Ether-soluble Factor Essential for Growth of Propionic Acid Bacteria, *J Bact.*, **33**: 227 (1937).

——— and C. H. Werkman: The Propionic Bacteria on the Mechanism of Glu Dissimilation, *Jour. Biol. Chem.*, **105**: 63 (1934).

and ———: CIII. Pyruvic Acid in the Dissimilation of Glucose by the Pro-
nic Acid Bacteria, *Biochem. Jour.*, **28**: 745 (1934).
ınd ———: The Isolation and Possible Intermediary Role of Formaldehyde in
Propionic Acid Fermentation, *Jour. Bact.*, **30**: 652 (1935).
ınd ———: X. The Utilization of $CO_2$ in the Dissimilation of Glycerol by
Propionic Acid Bacteria, *Biochem. Jour.*, **30**: 48 (1936).
ınd ———: LXXXVIII. Mechanism of Glucose Dissimilation by the Pro-
ic Acid Bacteria, *Biochem. Jour.*, **30**: 618 (1936).
nd ———: The Utilization of Carbon Dioxide by the Propionic Acid Bacteria,
hem. Jour., **32**: 1262 (1938).
W. P. Wiggert, and C. H. Werkman: The Fermentation of Phosphate Esters
he Propionic Acid Bacteria, *Enzymologia*, **2**: 373 (1938).

## Some Patents

ı, J. M.: Propionic Acid Fermentation by the Use of Mixed Strains of Pro-
ic Bacteria, U.S. Patent 1,865,146, June 28, 1932.
Method of Accelerating the Propionic Acid Fermentation, U.S. Patent
0,130, May 23, 1933.
I. R.: Propionic Acid Fermentation of Fructose-containing Mashes, U.S.
nt 1,946,447, Feb. 6, 1934.
ınd P. W. Wilson: Production of Propionic Acid, U.S. Patent 1,932,755,
31, 1933.
P. W.: Propionic Acid Fermentation, U.S. Patent 1,898,329, Feb. 21, 1933.
ff, C., and P. W. Wilson: Propionic Acid Fermentation, U.S. Patent 1,875,401,
. 6, 1932.

## CHAPTER 25

# THE PRODUCTION OF VITAMIN $B_{12}$

Vitamin $B_{12}$ was isolated from liver concentrate by Rickes et (1948*a*). Shortly thereafter Smith and Parker (1948), of Engla reported on the purification of the antipernicious anemia factor.

Since 1948, hundreds of papers concerned with vitamin $B_{12}$ have published. Reviews have been made by Goldeck (1953), Boettge (195 Smith (1954), Heinrich and Lahann (1954), Harris et al. (1954), Bri and Daft (1955), Williams et al. (1955), and others.

Vitamin $B_{12}$ is a water-soluble vitamin, commonly known as coba min. The related forms of $B_{12}$, which possess activity toward anim (vertebrates), are called the vitamin $B_{12}$ group, or cobalamin. This t vitamin $B_{12}$ group must be distinguished from the $B_{12}$-related compoun which promote growth activity only in the lower forms of life and wh are properly designated as members of a pseudo $B_{12}$ group (Briggs a Daft, 1955). Members of the latter group are closely related to but a not actually vitamins.

## STRUCTURE

Details concerning the very complex structure of vitamin $B_{12}$ ha been supplied by scientists from Merck & Co.; the Laboratory of Chem cal Crystallography, Oxford, England; the Department of Chemist University of California at Los Angeles; the Frick Laboratory, Prin ton, N.J.; the University Chemical Laboratory, Cambridge, England; t Glaxo Laboratories, Ltd., Greenford, England; and other laboratori Reference to reports appearing in the literature on the determination the structure of this vitamin has been made by Smith (1955), Briggs a Daft (1955), and others.

A complete structural formula for vitamin $B_{12}$ was reported by Hodgk et al. (1955). It is shown in Fig. 81.

## MICROORGANISMS PRODUCING

Vitamin $B_{12}$ is produced chiefly by actinomycetes and bacteria. Actin mycetes reported to be capable of producing vitamin $B_{12}$ or vitamin B like activity include a *Nocardia* species, and the following species of th genus *Streptomyces: albidoflavus, antibioticus, aureofaciens, colombie sis, griseus, olivaceus,* and *roseochromogenus.* Bacteria include *Aerobact aerogenes; Bacillus megatherium; B. subtilis; Clostridium butyricum;*

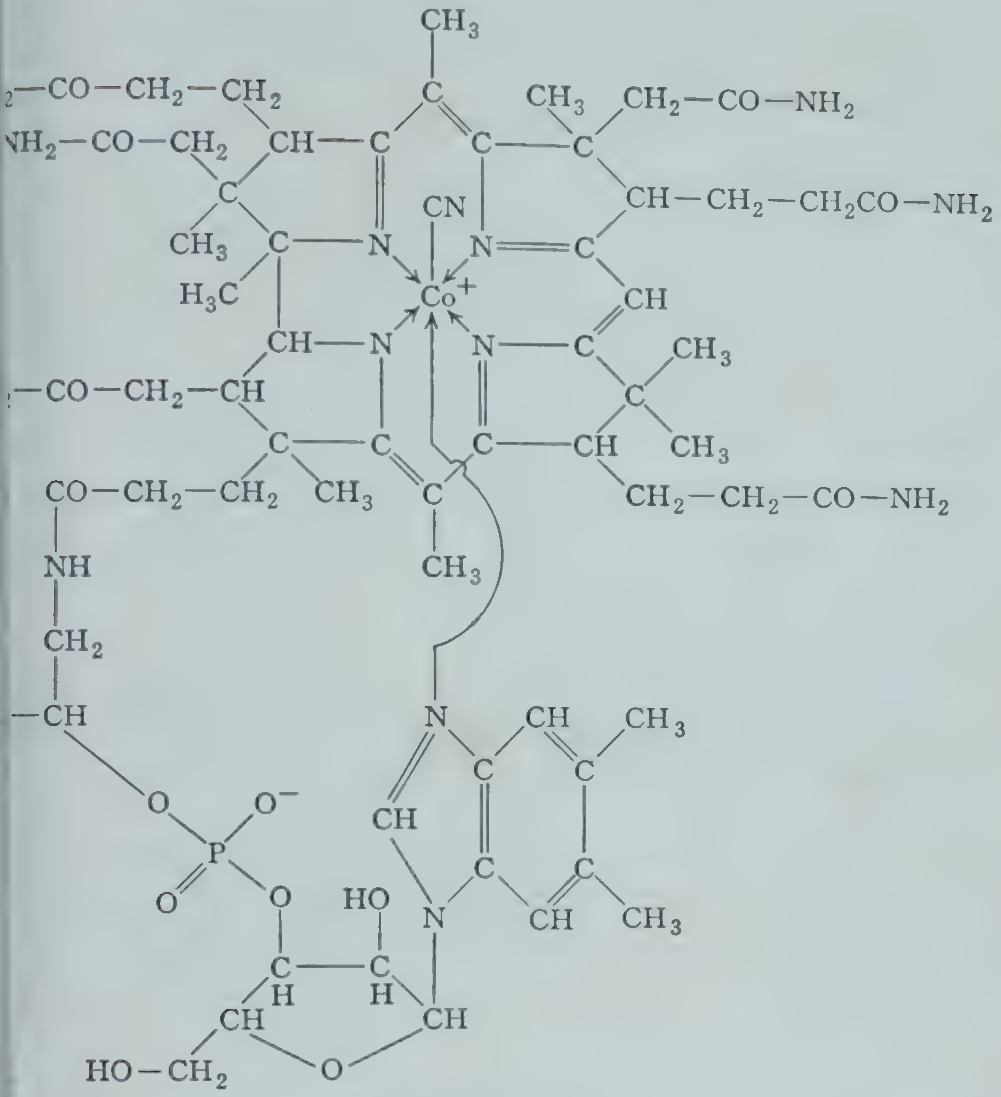


FIG. 81. Vitamin $B_{12}$.

*rium; Cl. flabelliflerum; Cl. tetaromorphum; Escherichia coli;* the
*acterium* species *acetylicum, acidificum, aquatile, arborescens,*
*ns, esteroaromaticum, flavescens, solare,* and *suaveolens; Lacto-*
*s arabinosus; L. casei; Mycobacterium phlei; M. smegmatis; M.*
*ilosum; Propionibacterium freudenreichii; P. shermanii; P. zeae;*
*s vulgaris; Pseudomonas* species; *Serratia marcescens; Staphylo-*
*aureus;* and *Streptococcus faecalis* (Darken, 1953). Certain spe-
*Rhizobium* are able to synthesize vitamin $B_{12}$ (Levin, Funk, and
r, 1954).

luction of vitamin $B_{12}$ by 50 strains of *Streptomyces* isolated from
was studied by Janicki et al. (1954). The vitamin was produced
rn-steep–peptone medium by 86 per cent of the strains, usually in
rations of 0.1 to 0.3 μg. per ml.

e 135 lists some microorganisms which have received special study
ing vitamin $B_{12}$ production.

ls and yeasts are not promising sources of vitamin $B_{12}$.

Table 135. Some Microorganisms Which Produce Vitamin $B_{12}$

| Microorganisms | References |
|---|---|
| *Bacillus megatherium* NRRL B-938 | Lewis, J. G., K. Ijichi, N. S. Snell, and J. A baldi: *U.S. Dept. Agr., Bur. Agr. and Chem., Mimeographed Circ. Ser., AIC* October, 1949.<br>Garibaldi, J. A., K. Ijichi, N. S. Snell, and Lewis: *Ind. Eng. Chem.,* **45**: 838 (1953). |
| *Flavobacterium devorans* NRRL B-54 | Hall, H. H., and H. M. Tsuchiya: U.S. P 2,561,364, July 24, 1951. |
| *F. solare* | Petty and Matrishin: *Bact. Proc., 49th Me* 1949, p. 47. Petty: U.S. Patent 2,515,135, 11, 1950. |
| *Proteus vulgaris; Pseudomonas* sp.; *Streptococcus bovis* | Hodge, H. M., C. T. Hanson, R. J. Allg *Ind. Eng. Chem.,* **44**: 132 (1952). |
| *Propionibacterium freudenreichii* A.T.C.C. 6207 | Hargrove, R. E., and A. Leviton: *Bact. Proc., Meeting,* 1952, p. 21.<br>Leviton, A., and R. E. Hargrove: *Ind. Chem.,* **44**: 2651 (1952). |
| *Streptomyces aureofaciens* | Pierce, Page, Stokstad, and Jukes: *Jour. Chem. Soc.,* **71**: 2952 (1949). |
| *S. griseus* | Rickes, E. L., N. G. Brink, F. R. Koniuszy, T Wood, and K. Folkers: *Science,* **108**: (1948).<br>Hendlin and Ruger: *Science,* **111**: 541 (1950)<br>Tarr, H. L. A.: *Can. Jour. Tech.,* **29**: 391 (19<br>Wood and Hendlin: U.S. Patent 2,595,499, M 6, 1952. |
| *S. olivaceus* NRRL B-1125 | Hall, H. H., R. G. Benedict, C. F. Wie C. E. Smith, and R. W. Jackson: *Appl. Mic biol.,* **1**: 124 (1953).<br>Hester, A. S., and G. E. Ward: *Ind. Eng. Che* **46**: 238 (1954).<br>Pfeifer, V. F., C. Vojnovich, and E. N. Heg *Ind. Eng. Chem.,* **46**: 843 (1954). |
| *Serratia marcescens* | Hill and Branion: *Poultry Sci.,* **32**: 367 (1953 |

## PRODUCTION BY STREPTOMYCES OLIVACEUS

*S. olivaceus* has been described by Waksman (1919), by Breed et (1957), and by Waksman and Lechevalier (1953). It has the ability peptonize casein, liquefy gelatin, reduce nitrate to nitrite, and hydrol starch. Its optimum temperature is reported to be 25°C. Strain NR B-1125, which has been used for the commercial production of vitan as isolated by the Northern Utilization Research and Development on of the U.S. Department of Agriculture.

ails concerning the production of vitamin $B_{12}$ by *S. olivaceus* , B-1125 have been reported by Hall and associates (1951, 1953); r and Ward (1954); Pfeifer, Vojnovich, and Heger (1954); and .

**culum Preparation.** The culture is maintained and sporulates well nts of Bennett's agar (Jones, 1949), which has the composition below:

| | |
|---|---|
| Yeast extract | 1.0 g. |
| Beef extract | 1.0 g. |
| N-Z-Amine A * | 2.0 g. |
| Glucose | 10.0 g. |
| Agar | 15.0 g. |
| Distilled water | 1,000 ml. |

Adjust pH to 7.3 with NaOH

* Enzymatic hydrolyzate of casein.

es for inoculation purposes are prepared by growing the organism nnett's agar for 4 to 6 days at 28°C. These may be used immedi- or may be stored for several weeks at refrigeration temperature ised.

umber of different media may be used for building up the inoculum. edium contains 1 per cent corn-steep liquor (or 0.5 per cent solids) 5 per cent dextrose (Milner, 1953). Often the composition of the ation medium is identical to that used for the final fermentation. les are given in Table 136.

TABLE 136. INOCULATION AND FERMENTATION MEDIA

| | Medium 1 * | Medium 2 † |
|---|---|---|
| Dextrose, % | 0.5 | 1.0 |
| Corn-steep-liquor solids, % | 0.5 | |
| Distillers' solubles or soybean meal *. | ... | 4.0 |
| Cobalt chloride ($CoCl_2 \cdot 6H_2O$), p.p.m. | 2.0 | 2–10 |
| Calcium carbonate, % | ... | 0.5 |
| Soybean oil, % | 0.1 | 0.1 |
| Adjust pH to | 7.0 | 7.0 |

racted and unheated.

F. Pfeifer et al., *Ind. Eng. Chem.*, **46**: 843 (1954). Copyright 1954 by the an Chemical Society and reprinted by permission of the copyright owner.

liquid medium is inoculated with spores and incubated with aera- agitation for about 48 hr. at 28°C. For shake-flask fermentations, may be developed on rotary or reciprocating shakers; for deep- rmentations, they may be built up in aerated flasks or bottles. 5 per cent by volume of inoculum is used to inoculate the next volume in each case (Hall et al., 1953).

**Flow Diagram.** Figure 82 shows a flow diagram for the prod of vitamin $B_{12}$ with *S. olivaceus.*

**pH.** The pH may be adjusted to 7 to 7.5 before sterilization and or may not be controlled during fermentation. Figure 83 show changes in pH, sugar, and vitamin $B_{12}$ during a typical fermentati which the pH was not controlled.

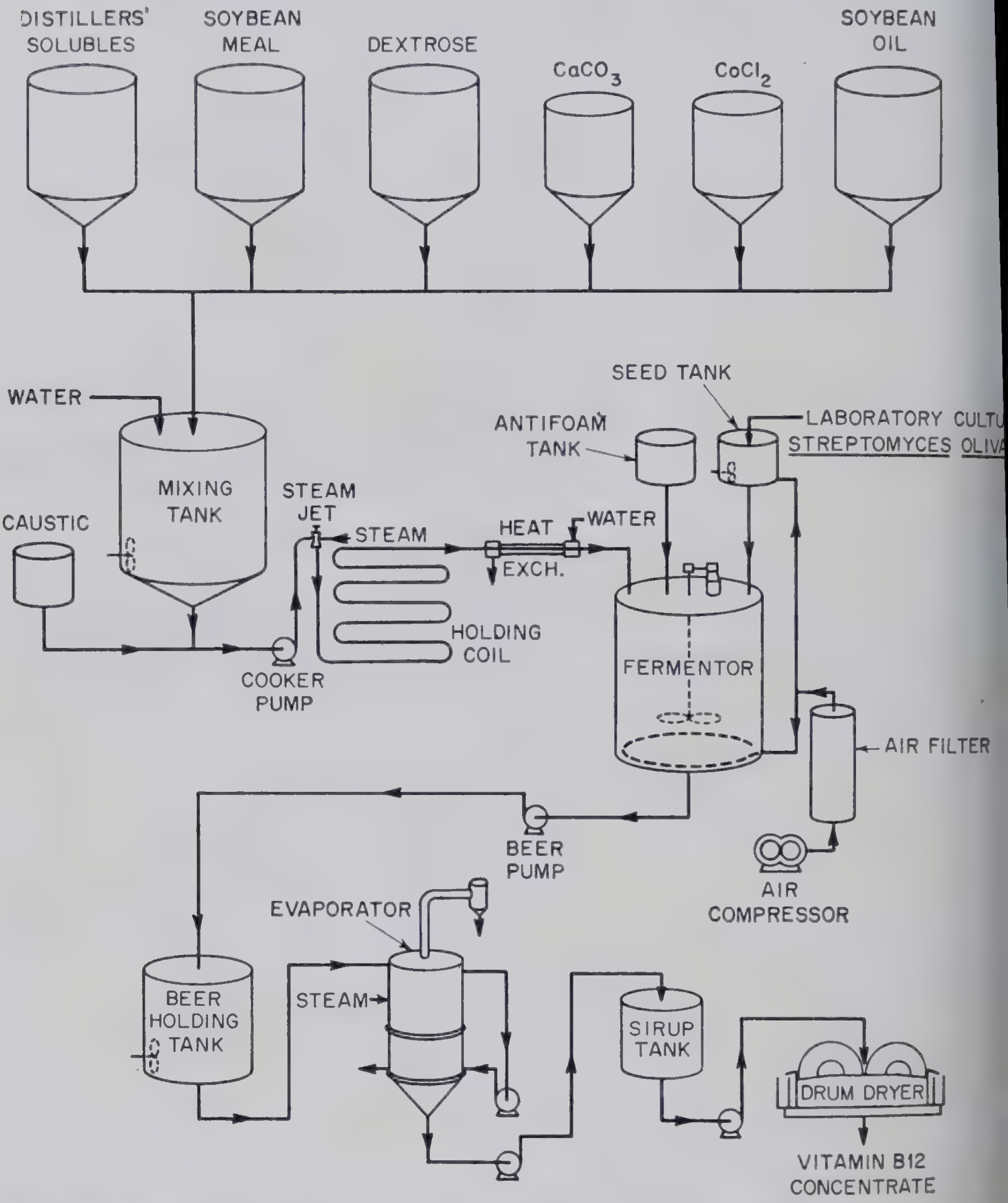
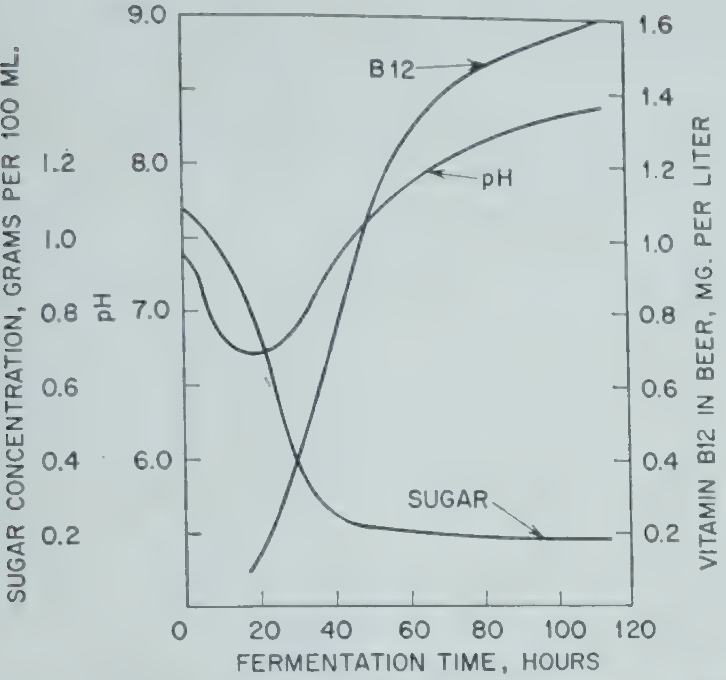

DISTILLERS' SOLUBLES
SOYBEAN MEAL
DEXTROSE
$CaCO_3$
$CoCl_2$
SOYBEAN OIL
WATER
MIXING TANK
CAUSTIC
STEAM JET
STEAM
HEAT EXCH.
WATER
HOLDING COIL
COOKER PUMP
ANTIFOAM TANK
SEED TANK
LABORATORY CULTU
STREPTOMYCES OLIVA
FERMENTOR
AIR FILTER
AIR COMPRESSOR
BEER PUMP
EVAPORATOR
STEAM
BEER HOLDING TANK
SIRUP TANK
DRUM DRYER
VITAMIN B12 CONCENTRATE

Fig. 82. Flow sheet for production of vitamin $B_{12}$ by fermentation. [*Courtesy V. F. Pfeifer, C. Vojnovich, and E. N. Heger, Ind. Eng. Chem.,* **46**: 843 (19 *Copyright* 1954 *by the American Chemical Society and reprinted by permission the copyright owner.*]

**Sterilization.** Media and materials used for the production of vi min $B_{12}$ must be sterilized, along with the fermentation tanks and asso ated lines and connections. Batch or continuous sterilization may be us for media. Pfeifer and associates found that it was necessary to he



Changes in medium during vitamin $B_{12}$ fermentation. [*Courtesy of V. F. C. Vojnovich, and E. N. Heger, Ind. Eng. Chem.*, 46: 843 (1954). *Copyright the American Chemical Society and reprinted by permission of the copy- wner.*]

for 1.5 to 2 hr. at 250°F. to effect sterilization in batch operations. enough copper was dissolved from copper tanks at low pH values ; this treatment to inhibit the growth of *S. olivaceus*, it was essential ust the pH of the medium to 7.0 before heating in order to obtain ctory results. The use of continuous sterilization at a temperature °F. and a retention time of 13 min. was acceptable even at a low r fermentations carried out in copper, stainless-steel, or steel tors.

**perature.** Optimum yields of vitamin $B_{12}$ from *S. olivaceus* B-1125 were obtained at a temperature of 80°F.; however, the f 78 to 82°F. is satisfactory.

**ation and Agitation.** Vigorous agitation of the fermentation is d for best yields. Sterile air should be passed through at a rate ut 0.25 to 0.5 volume per volume of medium per minute. Accord- Pfeifer et al., the minimum oxygen absorption rate (determined by fite oxidation method) should be 1.0 mM. of oxygen absorbed per e per liter.

**overy.** Vitamin $B_{12}$ is associated largely with the mycelium of *aceus* during about the first 60 hr. of fermentation (refer to Fig. onsequently, most of the activity may be recovered with the um by centrifuging or filtering the whole beer after 70 to 90 hr. lids obtained in this manner may be dried directly in an atmos- -compartment type of drier at 120°F. Recovery yields of 95 per ay be achieved.

ther method for obtaining a concentrate involves reducing the pH beer to 5 with sulfuric acid, heating the mixture to boiling to liberate the vitamin, centrifuging or filtering to remove the unde solids, evaporating the centrifugate or filtrate under vacuum, and to a sirup. Sodium sulfite in a concentration of about 100 p.p.m. m added to the mixture after reducing the pH in order to protect the vi during subsequent processin concentrate prepared in this ner may be expected to conta to 30 mg. of vitamin $B_{12}$ per p according to Pfeifer and assoc (1954). By using the mycelium arated from whole, fresh beer, pending it in water, reducing pH of the suspension to 5 with furic acid, heating it to boi centrifuging or filtering, evapo ing the liquor to a sirup under uum, and drying, a concent containing 100 mg. of vitamin per pound may be obtained.

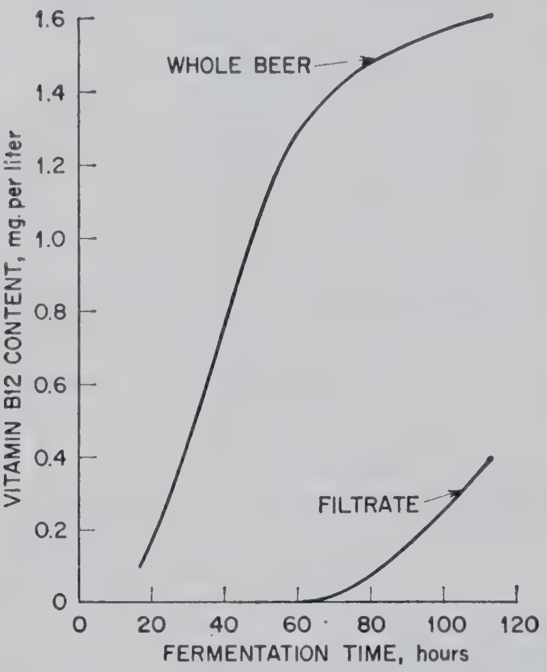


Fig. 84. Distribution of vitamin $B_{12}$ in fermented beer. [*Courtesy of V. F. Pfeifer, C. Vojnovich, and E. N. Heger, Ind. Eng. Chem.,* **46**: 843 (1954). *Copyright* 1954 *by the American Chemical Society and reprinted by permission of the copyright owner.*]

Concentration may be acc plished through the use of lo tube vertical evaporators or v uum pans or otherwise, followed the use of steam-heated doub drum driers of an atmospheric ty (Hester and Ward, 1954).

The dried product, which co tains about 5 per cent of moistu is passed through a hammer m mixed to obtain uniformity, a packaged. The vitamin $B_{12}$ content may vary from 10 to 30 mg. per in the final product.

## PRODUCTION OF COBALAMIN BY BACILLUS MEGATHERIUM

A commercial process for the production of the vitamin $B_{12}$ comp through the use of *Bacillus megatherium* was reported by Gariba Ijichi, Snell, and Lewis (1953). This process, described in prelimina form by Lewis et al. (1949) and by Garibaldi and associates (1951), discussed briefly below.

Strains of *B. megatherium* which included NRRL B-938, B-938 R phage-resistant variant), and B-1366 to B-1372 were used for cobalam synthesis. One of these strains, NRRL B-938, was described by Smi Gordon, and Clark (1946).

The strains of *B. megatherium* studied were relatively large in si measuring 2 to 3 by 4 to 6 μ and larger. Such cells are sufficiently la to be recovered in a yeast centrifuge.

**ation of Cobalamin-producing Strains.** A special agar medium Table 137) was used for the isolation. Samples of soil were diluted

TABLE 137. COMPOSITION OF MEDIA *

| Constituent | Medium,† g./liter | | | | | | |
|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G |
| : | | | | | | | |
| ied | 10 | 50 | 50 | | | | |
| eet molasses | ... | ... | ... | 50 | 62 | | |
| trus molasses | ... | ... | ... | ... | ... | 12 | |
| whey | ... | ... | ... | ... | ... | ... | 50 |
| xtract | ... | 2.5 | 2.5 | | | | |
| acid (anhyd.) | 1.2 | 11.7 | 0.5 | | | | |
| | 15 | | | | | | |
| HPO$_4$ | 0.4 | 2.1 | 4.2 | 4.2 | 8.0 | 2.0 | 1.0 |
| | 0.4 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | |
| | 0.08 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | |
| | ... | ... | ... | ... | ... | ... | 0.9 |
| pH 7 | | | | | | | |
| | Mg./liter | | | | | | |
| chloride) | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| chloride) | ... | 20 | 20 | 50 | 50 | 20 | 20 |
| chloride) | 15 | 15 | 15 | 15 | 50 | 15 | 15 |
| chloride) | 10 | 5 | 5 | 5 | 5 | 5 | 5 |
| chloride) | 10 | 5 | 5 | 5 | 5 | 5 | 5 |
| chloride) | 1 | 2 | 2 | 2 | 5 | 2 | 2 |

rtesy of J. A. Garibaldi, K. Ijichi, N. S. Snell, and J. C. Lewis, *Ind. Eng.* **45**: 838 (1953). Copyright 1953 by the American Chemical Society and re- by permission of the copyright owner.

iants of basic media: $B_a$ contained no yeast extract, $B_c$ contained 5.8 g. of ɜid per liter, $C_a$ contained no yeast extract, $C_b$ and $C_c$ were like $C_a$ but con- ).05 g. and no citric acid, respectively, per liter.

ater to yield 10 to 30 surface colonies per plate. The seeded agar were incubated at 30°C. for 24 hr., then were opened and per- to dry at 50°C. for several hours in order to help prevent the ing of the colonies during the next procedure, which consisted of ; a layer of cobalamin assay agar (1.5 per cent) seeded with *Lacto- is leichmannii* A.T.C.C. 4797. The plates were next incubated at for 18 to 24 hr. *L. leichmannii* grew in very faint halos around those es which produced cobalamin. Cultures were picked from such col- and purified by additional plating. Stock cultures were carried on it agar and stored at refrigeration temperature.

ins isolated from medium A were cultivated in 100-ml. portions of n B in shake flasks until maximum growth occurred. At this liquots of the whole culture were assayed for cobalamin. It was ossible to screen organisms and to study the most promising ones.

**Media and Nutrients.** Examples of media which may be used f production of cobalamin are shown in Table 137. Sucrose, glucose, tose, and raffinose give good yields of cobalamin. However, corn s corn sirup, invert sugar, cane molasses (beet, cane, and citrus), st and certain polyhydric alcohols may be used also as carbon sources. optimum concentration of sucrose for cobalamin production is 5 per cent.

Nitrogen may be supplied in organic form by proteins, enzyme-dig proteins, amino acids, yeast extract, asparagine, or urea and in inorg form by diammonium phosphate, ammonium citrate, ammonium ace ammonium sulfate, ammonium nitrate, ammonium hydroxide, or monia. The minimal optimal range of nitrogen is 1,700 to 2,200 mg liter for the growth and for cobalamin production by *B. megathe* NRRL B-938.

Citric acid was added to media to increase the solubilities of the s and to serve for buffer purposes.

**Conditions of Propagation.** The following conditions were used Garibaldi et al. (1953), except as noted in Table 138. The ferme described by Humfeld (1947) was used for 10-liter amounts of medi however, a few runs were carried out in the 150-liter propagator descri by Garibaldi and Feeney (1949) and in the 1-liter propagator described Feustel and Humfeld (1946). The media, which contained 5 per c sugar, were sterilized for 45 min. at 15 lb. steam pressure. The inocul for a propagator was grown on the same medium as that used in propagator. The amount used represented 5 to 10 per cent of the volu of the propagation medium.

*pH.* The pH may be varied from 7.5 to 5.5. However, a pH of 6.5 7.0 was optimum for at least one strain (NRRL B-938) of *B. me therium*. The pH may be maintained at a relatively constant level by controlled addition of ammonia, ammonium hydroxide, sodium hydroxi or potassium hydroxide. The pH may also be controlled through the of buffers, such as phosphoric or citric acid or their water-soluble salts.

*Temperature.* A temperature range of 35 to 40°C. may be used for fermentation, although one of 35°C. is favored (refer to Fig. 85). T maximum temperature for the growth of *B. megatherium* NRRL B-938 42 to 44°C. Cells grown at 40°C. are smaller than those at 30 and 35° (Garibaldi et al., 1953).

*Aeration.* Air is supplied for the growth of the organism and for agit tion of the medium. The amount supplied may vary from 0.1 to 2 volum per volume of medium per minute. Mechanical agitation and fine aer tion are helpful. The air must be sterilized before use.

*Defoaming agents.* A sterilized defoaming agent may be required control excessive foaming during fermentation. Monoglyceride in lard c octadecanol diluted with purified mineral or lard oil, DC Antifoam A a Vegifat Y are examples of suitable defoaming agents.

*Time.* Good yields of cells and vitamin $B_{12}$ are usually obtained 6 to 12 hr.

| | | | | growth | | cells, g. § | |
|---|---|---|---|---|---|---|---|
| 42 | pH uncontrolled | B § | 1 | 7 | 7–8 | 45 | 0.38 |
| 110 | 10% sugar, 37°C. | C | 2 | 11 | ≧11 | 48 | 0.91 |
| 120 | 10% sugar, 37°C. | C | 1 | 12 | ≧13 | 43 | 0.80 |
| 121A | Heavy precipitation of salts due to lack of citrate, light inoculum from shake culture | C § | Long | >24 | .......... | 15 | |
| 121B | As above; some precipitation due to low citrate | C ‡ | 6 | 18 | >18, ≦23 | 46 | 0.89 |
| 124B | Beet-molasses medium | E | 2 | 8 | 11 | .. | 0.72 |
| 126A | 30°C. | E | 2.5 | 9 | ≧11 | .. | 0.66 |
| 126B | 37°C. | E | 2 | 8 | 10 | .. | 0.73 |
| 127A | Control on simplified medium | C | 1 | 7.5 | 9 | 54 | 0.56 |
| 127B | Medium as in 127A, without yeast extract | C † | 8 | 14.5 | >14.5, ≦23 | 48 | 0.57 |
| 131A | Sugar (purified) fed in 1% increments | C | 3 | 11 | 11 | 29 | 0.45 |
| 131B | As above, but sugar as citrus molasses | F | 2.5 | 10 | 13 | 51 | 0.66 |
| 133A | 0.58% citrate medium | B § | 2 | 8 | ≧10 | 60 | 0.66 |
| 133B | 0.05% citrate medium | C | 2 | 7 | 7–8 | 62 | 0.70 |
| 136A | Agitation 600 r.p.m. | C | 3 | >28 | ≧24 | 15 | 0.09 |
| 136B | Agitation 1,500 r.p.m. (normal) | C | 2 | 7 | 8–9 | 53 | 0.51 |
| 146A | 40% $O_2$ in aeration mixture | C | 2 | 8 | 9–10 | 55 | 0.59 |
| 146B | Normal aeration | C | 2 | 8 | 8–9 | 51 | 1.00 |
| 148A | 10.5% $O_2$ in aeration mixture | C | 1 | 7 | ≧9 | 48 | 0.66 |
| 148B | Normal aeration | C | 1 | 7 | ≧9 | 51 | 0.62 |
| 150A | 2.1% $O_2$ in aeration mixture | C | 1 | >11, ≦24 | >11, ≦24 | 18 | 0.29 |
| 150B | Normal aeration | C | 1 | 7 | 8 | 54 | 1.02 |
| 188A | 0.1 vol. air/vol. medium/min. | B | 1 | 8 | 10 | 55 | 1.06 |
| 188B | 1 vol. air/vol. medium/min. (normal) | B | 1 | 7 | 8 | 54 | 1.10 |
| 220 | pH range 5.8–6.5; 150-liter propagator; inoculum in lag growth phase | B | 0 | 7.5 | >8, ≦18 ¶ | 55 | 0.77 |

---

* Courtesy of J. A. Garibaldi, K. Ijichi, N. S. Snell, and J. C. Lewis, *Ind. Eng. Chem.*, **45**: 838 (1953). Copyright 1953 by the American Chemical Society and reprinted by permission of the copyright owner.

† See Table 137.

‡ Lag time is interval between observed and ideal (no lag) exponential growth rate.

§ Estimated from dry-washed cell weights, bacterial turbidity, and centrifuged fresh cell volumes.

¶ Aeration stopped and culture cooled to 20°C. at 8 hr.; cobalamin yield at 8 hr. was 72% of that at 18 hr.

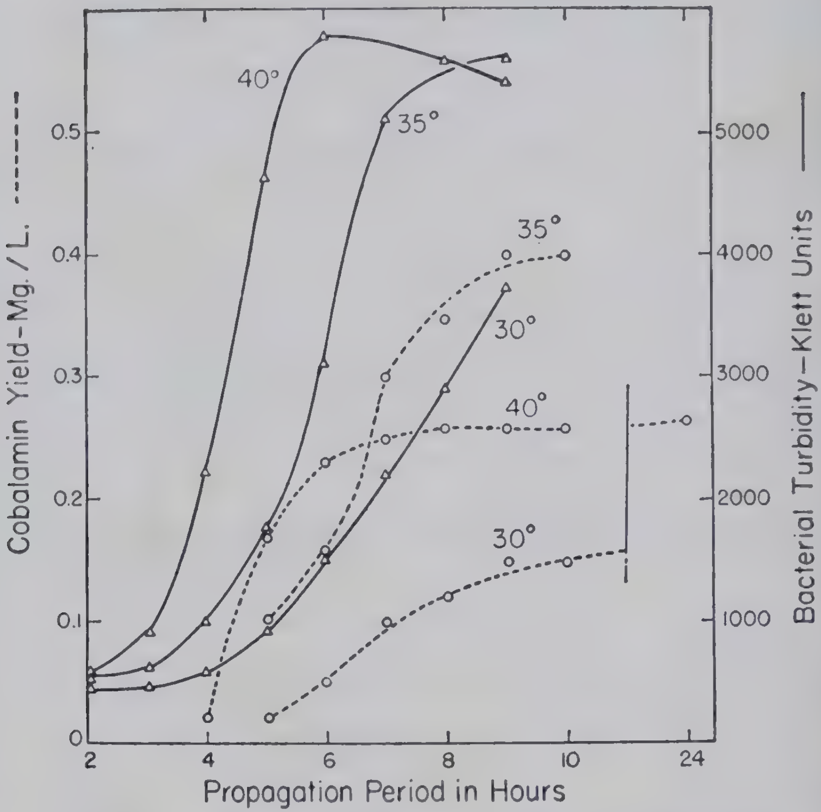


FIG. 85. Effect of temperature on growth and cobalamin biosynthesis on simplifi medium. Cells grown at 40°C. were much smaller than those grown at 35 and 30° [*Courtesy of J. A. Garibaldi, K. Ijichi, N. S. Snell, and J. C. Lewis, Ind. Eng. Che* 45: 838 (1953). *Copyright* 1953 *by the American Chemical Society and reprint by permission of the copyright owner.*]

**Recovery.** The general method of recovery is similar to that used f the vitamin $B_{12}$ produced by *Streptomyces olivaceus.* The cells, whi contain essentially all of the vitamin, may be separated from the liqu by centrifugation, filtration, or decantation and used as a feed suppleme with or without drying. If the vitamin is desired for medical purposes, suspension of the washed cells in water is heated to 100°C. to provo autolysis of the cells and liberation of the vitamin into the aqueous phas The vitamin is concentrated and purified.

**Yields.** Data concerning special conditions of propagating *B. meg therium* NRRL B-938 under forced aeration, the media used, the hou required for maximum growth and cobalamin, and the yields of bacteri cells and cobalamin per 100 g. of sugar are presented in Table 138.

**Assay.** It is highly important that the method, or methods, used f assay differentiates between vitamin $B_{12}$ and pseudo vitamin $B_{12}$. Severa compounds similar to the vitamin exist which possess little or no growt promoting activity for animals.

Chaiet et al. (1954) determined the vitamin $B_{12}$ content of feed suppl ments and the effect of pseudo vitamin $B_{12}$, using a number of differe assay methods. They found that the microbial and the spectrophot metric assay methods did not differentiate the true and pseudo vitam $B_{12}$ and consequently may lead to erroneous results of large magnitu

| Manufacturer | Label, mg. $B_{12}$/lb. | Isotope dilution | *Lactobacillus leichmannii*, U.S.P. |  | Microbial-cup assay |  | Spectrophotometric |  | Chick-growth assay |
|---|---|---|---|---|---|---|---|---|---|
|  |  |  | Titrimetric | Turbidimetric | LLD | *Escherichia coli* | Rudkin and Taylor | Fisher |  |
| A | 13.5 | 11.6 | 13.5 | 13.7 | 13.3 | 10.8 | 8.0 | 0.0 |  |
| B | 113.0 | <0.3 | 77.0 | 58.5 | 98.0 | 220.0 | 11.6 | 113, 71, 94 | <4.0 |
| $B_1$ | 25.0 | 0 | ..... | ..... | ..... | ..... | 3.3 | 25 |  |
| C | 12.5 | 8.7 | 10.0 | 10.0 | 11.0 | 9.8 | 6.7 | 3.4 |  |
| D | 12.5 | 8.5 | 11.0 | 9.0 | 8.0 | 8.5 | 7.7 | 10.3 |  |
| E | 12.5 | 11.4 | 12.5 | 11.0 | 11.5 | 10.5 | 7.3 | 14.5 |  |
| F | 10.0 | 8.8 | 9.5 | 11.5 | 9.5 | 9.4 | 12.5, 9.6, 6.0 | 91.0 |  |
| G | 10.0 | 8.6 | 8.5 | 10.0 | 9.0 | 6.9 | 4.2 | 0.0 |  |
| H | 6.0 | 6.3 | 8.2 | 7.0 | 7.3 | 6.4 | 4.2 | 0.0 |  |
| I | 6.0 | 5.5 | 6.5 | 6.0 | 4.5 | 9.0 | 3.2 | 0.0 |  |
| J | 6.0 | 4.0 | 3.8 | 4.0 | 4.8 | 4.5 | 1.3 | 2.9 |  |
| K | 6.0 | 5.6 | 9.6 | 8.5 | 13.6 | 8.3 | 4.3 | 2.0 |  |
| L | 3.0 | 3.0 | 4.2 | 3.9 | 4.7 † | 3.7 | 1.8 | 3.7 | 2.6 ± 0.5 |
| Sewage product.. | ..... | 1.6 | 1.7 | ..... | ..... | 4.1 | .......... | .......... | 1.1 ± 0.2 |
| Sewage-product extract... | ..... | 11.3 | 12 |  |  |  |  |  |  |
| Average ‡.. | 8.9 | 7.5 | 8.3 | 8.6 | 8.8 | 8.4 | 5.3 | 3.7 § |  |

---

* Courtesy of L. Chaiet, T. Miller, and A. E. Boley, *Jour. Agr. Food Chem.*, 2(15): 784 (1954). Copyright 1954 by the American Chemical Society and reprinted by permission of the copyright owner.

† The presence of penicillin in this preparation interfered with the LLD assay. The use of penicillinase eliminated interference.

‡ Average includes products A through L and excludes products B and $B_1$.

§ This average does not include product F, which is abnormally high.

that the vitamin $B_{12}$ content of feed supplements was somewhat low
an isotope dilution assay than by the U.S.P. microbial assay method
that one commercial product contained no vitamin $B_{12}$.

Study of the data contained in Table 139 will reveal interesting i
mation concerning several different assay methods used to determin
vitamin $B_{12}$ in commercial animal protein factor (APF) products.

**Uses.** Vitamin $B_{12}$ has a number of important applications in
fields of animal and human nutrition, medicine, and microbiology. W
incorporated into stock feed at the rate of 10 to 15 mg. per ton, it impr
the utilization of vegetable proteins by poultry, swine, and other ani
There is an increase in body weight, owing partly to increased food int
It maintains a satisfactory level of hatchability of hens' eggs. It app
to stimulate the appetite and growth of children with retarded growth
is effective in the treatment of pernicious anemia and some of the neu
logical disorders and the degeneration of the spinal cord associated w
this disease. It is used in the treatment of nutritional macrocytic ane
megaloblastic anemia of infancy, and tropical and nontropical sprue.
is essential for the growth of *L. lactis* (Dorner) and is a growth factor
other lactobacilli, mutants of *Escherichia coli*, and other microorganis

**Further Reading.** The interested reader is referred to the publicati
listed below for additional information.

### Some References concerning Vitamin $B_{12}$

Ambrose, A. M., and F. DeEds.: Comparative Studies on the Toxicity of Wash
Cells of *Bacillus megaterium* and Brewers' Yeast, *Jour. Am. Pharm. Assoc.,*
*Ed.,* **43**(3): 185–187 (1954).

Anonymous: "Vitamin $B_{12}$, Annotated Bibliography," Merck & Co., Inc., 1954.
three parts.)

Bessey, O. A., H. J. Lowe, and L. L. Salmon: Water-soluble Vitamins, *Ann. R*
*Biochem.,* **22**: 545–555 (1953).

Boettge, K.: *Arzneimettel-Forsch.,* **3**: 348 (1953).

Breed, R. S., E. G. D. Murray, and A. P. Hitchens (eds.): "Bergey's Manual
Determinative Bacteriology," 7th ed., The Williams & Wilkins Company, Bal
more, 1957.

Briggs, G. M., and F. S. Daft: Water-soluble Vitamins, Part I, *Ann. Rev. Bioche*
**24**: 339–358 (1955). (Contains extensive bibliography.)

Brink, N. G., F. A. Kuehl, Jr., and K. Folkers: Vitamin $B_{12}$: The Identification
Vitamin $B_{12}$ as a Cyano-Cobalt Coordination Complex, *Science,* **112**: 354 (Se
29, 1950).

Chaiet, L., T. Miller, and A. E. Boley: Determination of $B_{12}$ Content of Feed Supp
ments and Effect of Pseudo Vitamin $B_{12}$, *Jour. Agr. Food Chem.,* **2**(15): 784–
(1954).

Darken, M.: Production of Vitamin $B_{12}$ by Microorganisms and Its Occurrence
Plant Tissues, *Botan. Rev.,* **19**(2): 99–130 (1953). (Article contains 155 r
erences.)

Emerson, G., and K. Folkers: Water-soluble Vitamins, *Ann. Rev. Biochem.,* **20**: 5
574 (1950).

Feustel, I. C., and H. Humfeld: A New Laboratory Fermenter for Yeast Producti
Investigations, *Jour. Bact.,* **52**(2): 229–235 (1946).

Garibaldi, J. A., and R. E. Feeney: Subtilin Production, *Ind. Eng. Chem.,* **41**:
(1949).

———, K. Ijichi, J. C. Lewis, and J. McGinnis: Fermentation Process for Producti
of Vitamin $B_{12}$, U.S. Patent 2,576,932, Dec. 4, 1951.

———, N. S. Snell, and J. C. Lewis: *Bacillus megatherium* for Biosynthesis
Cobalamin, *Ind. Eng. Chem.*, **45**: 838–846 (1953).

., H.: *Folia Haematol.*, **71**: 403 (1953).

. H.: The Fermentative Production of Vitamin $B_{12}$ in Grain Stillage and
er Materials, *Proceedings of the 6th Distillers Feed Conference*, Distillers
d Research Council, Inc., Cincinnati, Ohio, Mar. 15, 1951.

R G. Benedict, C. F. Wiesen, C. E. Smith, and R. W. Jackson: Studies on
amin $B_{12}$ Production with *Streptomyces olivaceus*, *Appl. Microbiol.*, **1**(3):
-129 (1953).

J. C. Benjamin, H. M. Bricker, R. J. Gill, W. C. Haynes, and H. M. Tsuchiya:
urvey for Vitamin $B_{12}$-producing Microorganisms, *Soc. Am. Bacteriologists,
c. 50th, Baltimore*, May, 1950.

L. J.: "Vitamins in Theory and Practice," 4th ed., Cambridge University
ss, London, 1955.

ı, H. C. (ed.): "Vitamin $B_{12}$ und Intrinsic Factor," Ferd. Enke Verlag, Stutt-
, 1957.

and H. Lahann: *Zeit. Vitamin- Hormon- u. Fermentforsch.*, **6**: 126 (1954).

A. S., and G. E. Ward: Vitamin $B_{12}$ Feed Supplement, *Ind. Eng. Chem.*,
238–243 (1954).

H. M., C. T. Hanson, and R. J. Allgeier: Production and Evaluation of an
.F. Supplement Derived from Direct Bacterial Fermentation, 118*th Meeting
he American Chemical Society*, September, 1950.

ı, D. C., J. Pickworth, J. H. Robertson, K. N. Trueblood, R. J. Prosen, J. G.
ite, R. Bonnett, J. R. Cannon, A. W. Johnson, I. Sutherland, A. R. Todd, and
. Smith: Structure of Vitamin $B_{12}$, *Nature*, **176**(4477): 325–330 (1955).

S. R., L. Jasewicz, J. B. Pepinsky, and N. Porges: Activated Sludge as a
rce of Vitamin $B_{12}$ for Animal Feeds, *Sewage and Ind. Wastes*, **24**(1): 38–44
52).

———, and N. Porges: Vitamin $B_{12}$ in Activated Sewage Sludge, *Science*, **114**:
(1951).

l, H.: An Improved Laboratory-scale Fermenter for Submerged Culture In-
igations, *Jour. Bact.*, **54**(6): 689–696 (1947).

J., J. Pawelkiewicz, Stawicki, and K. Zodrow: *Acta Microbiol. Polon.*, **3**: 3
4).

A. W., and A. Todd: Vitamin $B_{12}$, *Endeavour*, **15**(57): 29–33 (January,
).

. L.: Fresh Isolates of Actinomycetes in which Presence of Sporogenous
al Mycelia Is a Fluctuating Characteristic, *Jour. Bact.*, **56**: 141–145 (1949).

. P., H. B. Funk, and M. D. Tendler: *Science*, **120**: 784 (1954).

. C.: Vitamin $B_{12}$ Production Speeded by a New Process, *U.S. Dept. Agr.,
Agr. and Ind. Chem., RAS* 170(*C*), February, 1954.

. Ijichi, N. S. Snell, and J. A. Garibaldi: Fermentation Process for Produc-
of Vitamin $B_{12}$, *U.S. Dept. Agr., Bur. Agr. and Ind. Chem., Mimeographed
. Ser., AIC* 254, October, 1949.

———, T. F. Sugihara, and J. A. Garibaldi: Rapid Mass Propagation of Some
eria of the *Bacillus* Genus, *Jour Agr. Food Chem.*, **1**(14): 897–899 (1953).

———, ———, P. A. Thompson, N. S. Snell, G. Alderton, and J. A. Garibaldi:
us *Bacillus* as a Source of Growth Stimulants, *Jour. Agr. Food Chem.*, **1**(19):
–1163 (1953).

R. T.: Private communication, U.S. Department of Agriculture, Northern
ization Research Branch, Peoria, Ill., 1953.

V. F., C. Vojnovich, and E. N. Heger: Vitamin $B_{12}$ by Fermentation with
*ptomyces olivaceus*, *Ind. Eng. Chem.*, **46**: 843–849 (1954).

. L., N. G. Brink, F. R. Koniuszy, T. R. Wood, and K. Folkers: Comparative
on Vitamin $B_{12}$ from Liver and from a New Source, *Streptomyces griseus*,
*nce*, **108**: 634 (1948*a*).

———, ———, ———, and ———: Crystalline Vitamin $B_{12}$, *Science*, **107**: 396–
(Apr. 16, 1948).

w, N. S., and M. A. Guzman: The Effect of Dietary Supplementation and

Administration of Vitamin $B_{12}$ and Aureomycin on the Growth of School dren, *Nutritional Symposium Series,* No. 7, National Vitamin Foundation New York, 1953, pp. 101–117.

Smith, E. L.: Water-soluble Vitamins, *Ann. Rev. Biochem.,* **23**: 245–260 (1954).

———: Isolation and Chemistry of Vitamin $B_{12}$, "Biochemistry of Vitamin Cambridge University Press, London, 1955, pp. 3–16.

——— and L. F. J. Parker: Purification of Anti-pernicious Anaemia Factor, *Bio Jour.,* **43**: 8 (May 29, 1948).

Smith, N. R., R. E. Gordon, and F. E. Clark: Aerobic Mesophilic Sporeforming teria, *U.S. Dept. Agr., Misc. Pub.* 559, 1946.

———, ———, and ———: Aerobic Sporeforming Bacteria, *U.S. Dept. Agr., M graph* 16, November, 1952.

Tarr, H. L. A.: Microbiological Formation of Vitamin $B_{12}$. I. Production in Press Liquid, *Can. Jour. Tech.,* **29**: 391–400 (1951).

Waksman, S. A.: *Soil Sci.,* **7**: 117 (1919).

——— and H. A. Lechevalier: "Guide to the Classification and Identification of Actinomycetes and their Antibiotics," The Williams & Wilkins Company, Ba more, 1953, p. 88.

Williams, R. T. (ed.): "The Biochemistry of Vitamin $B_{12}$," Biochemical Soci Symposia, No. 13, Cambridge University Press, London, 1955.

## Some Patents Relating to Vitamin $B_{12}$

Hargrove, R. E.: Process for the Manufacture of Vitamin $B_{12}$, U.S. Patent 2,715,6 Aug. 16, 1955.

Leviton, A.: Process for Microbiological Synthesis of Vitamin $B_{12}$ Active Substanc U.S. Patent 2,753,289, July 3, 1956.

Lindner, F., and K. Kuhn: Process for Recovering Vitamin $B_{12}$, U.S. Patent 2,793,1 May 21, 1957.

McCormick, J. R. D., and S. A. Muller: Recovery and Purification of Vitamin B U.S. Patent 2,745,787, May 15, 1956.

## CHAPTER 26

# MINOR BACTERIAL ACTIVITIES WITH INDUSTRIAL IMPLICATIONS

consideration of the possibilities of utilizing bacteria industrially
be unnecessarily incomplete if no mention was made of a number
esses, somewhat less sharply defined than those which have pre-
but which have well-grounded industrial implications. Through
research some of these minor bacterial activities may assume con-
ly greater significance, although they may not supply a basic
n for an industry and may not yield specific end products that can
chandised in pure form or in large quantity. Some of the bacterial
ses here may prove to be contributory and useful as adjuncts in
industries. Others may aid the student to a somewhat clearer
tion of processes long used in industry but in which the part played
robic agencies has not been well defined. In such cases it is still
ible to draw conclusions or make statements that can be regarded
l and final.

## ENZYME PRODUCTION BY BACTERIA

e enzyme production is recognized as a qualification of many
of bacteria, but the field of industrial application has not been
ghly investigated. As has been already pointed out in earlier
s, bacteria produce several enzymes, including those which can
yze carbohydrates, proteins, and fats and otherwise break down
x substances. The first step in the production of enzymes is to
cultures of bacteria that will grow profusely and produce most
ly the particular enzyme desired. Cultural conditions, such as
nposition and pH of the medium, the use of sterile equipment and
the temperature, the use of aeration, and other factors must be
ly worked out and controlled.

**ylases.** *Production Studies.* The production of amylase by bac-
as been studied by a number of scientists. Boidin and Effront [1]
gated amylase production by *Bacillus subtilis* and *B. mesentericus.*
stein (1939) and Schultz, Atkin, and Frey (1939) have described
s for preparing the media for use in producing bacterial amylase.
and Schaefer (1940) have investigated amylase production by

---

idin and J. Effront, U.S. Patent 1,227,374, May 22, 1917; and U.S. Patent
, May 22, 1917.

species of the genus *Bacillus* and two species of the genus *Sarcina;* T
and Hudson (1942), by *B. macerans* and *B. polymyxa;* and Hocke
and Herbert (1945), by *Clostridium acetobutylicum.* Bois and Sa
(1945) described the properties of the amylase produced by organism
the genera *Actinomyces* and *Phytomonas.* Beckord, Kneen, and L
(1945) have described the production of bacterial amylases on w
bran; and Beckord, Peltier, and Kneen (1946), on thin stillage. Pe
and Beckord (1945), Kneen and Sandstedt (1946), and Kneen and B
ord (1946) have studied a large number of bacterial isolates to deter
the quantity and quality of amylase produced.

*Wallerstein Procedure.* Wallerstein has described a procedure for p
ducing these bacterial enzymes, in particular, amylases. The medium u
may contain starch or the products of an enzymatically digested star
nitrogen from casein or from soybean or peanut cakes or other sour
hydrolyzed by means of enzymes or acids; mineral salts, such as ph
phates, and potassium, calcium, and magnesium salts plus traces of i
and manganese; and water. The medium, after filtration, sterilizati
and cooling, is inoculated with a strain of *B. subtilis.* Best results
obtained when the inoculated medium is poured to form shallow layers
trays (contained in large culture vessels, each of which may have
capacity for as much as 1,000 gal.), when the incubation temperature
about 30°C., and when aeration is supplied. Temperature and aerati
must be controlled carefully, the aeration being strongest at the begi
ning. Growth appears as bacterial films.

After the maximum enzyme content has been obtained, in perhaps
week, the culture medium containing the bacterial cells is centrifug
at approximately 14,000 r.p.m. to remove the bacteria. The resulta
liquor is preserved by incubation at a low temperature or by the use
chemical antiseptics.

*Waldmann Process.* Waldmann (1942) has described a process f
producing amylolytic enzymes from mashes containing carbohydrate a
nitrogen. Bacteria of the *B. mesentericus* or *B. subtilis* groups are used
inoculate mashes that contain at least 40 parts of carbohydrate-containi
substances, such as starch, soluble starch, or starch decomposition pro
ucts, for 1 part of assimilable nitrogen, such as ammonium sulfate, amm
nium lactate, or other ammonium salts. Buffers, as for example a mixtu
of $KH_2PO_4$ and $K_2HPO_4$, are added to the mash to maintain the pH lev
between 7 and 8 during the growth period. Air, in a fine state of subdiv
sion, is passed through the entire mash for 2 days, after which the enzym
solution is clarified and filtered. Concentration is effected in a vacuu
apparatus. It is stated that 1 kg. of the original enzyme solution is capa
ble of converting 1,000 to 3,000 kg. of starch.

Waldmann cited an example of the process in his patent. In a vat
1,000-hectoliter capacity are sterilized with pressure 700 hectoliters of
3 per cent solution of starch that contains 0.02 per cent of nitrogen
ammonium sulfate. Eight parts of a mixture of $KH_2PO_4$ and $K_2HPO$
of the correct proportions to produce and maintain a pH of 7.1 in th
solution, are added (during the cooling process) to the vat for each 1,0

f the starch solution. After the mash has been cooled to 28°C., it
ulated with amylase-forming bacteria of the *mesentericus* group
n aerated for 2 days, after which it is clarified and filtered.

vat may be constructed of aluminum or another metal protected
ating of rubber or otherwise treated so as to render it inactive.

*Production of Bacterial Amylases on Wheat Bran.* Beckord,
and Lewis have described a laboratory procedure for producing
al amylases on wheat bran, which is as follows:

ash may be prepared by adding 2.5 parts of dilute phosphate
(1.5 g. of $KH_2PO_4$ and 3.5 g. of $K_2HPO_4 \cdot 3H_2O$ per liter) to 1 part
ght of wheat bran (or other combination) in a suitable container
toclaving the mixture for 1 hr. at about 121°C. The pH of the
initially approximately 6. The sterilized mash is inoculated with
nsion of isolate No. 23 of *B subtilis*, incubated at 37°C. for 48 hr.,
n extracted with the phosphate buffer described above for 1 hr. at
There is added 1 ml. of a 20 per cent $CaCl_2 \cdot 2H_2O$ solution for
0 ml. of buffer. The precipitate and bran are separated by centri-
yielding a clear enzyme-containing extract.

conditions of production were studied in further detail by Beckord
s associates. They found that isolate no. 23 was the best of
bic bacilli known to hydrolyze starch; that neither the concentra-
the inoculum nor its age had appreciable influence on amylase
tion; that subculturing more than once in a liquid mash did not
te production; and that amylase production was dependent chiefly
he concentration of nutrient (wheat bran) present, the highest
f amylase per unit weight of bran resulting when 1 part of bran
parts of phosphate buffer was used.

advantages of producing amylases on wheat bran are (1) the
urface area available for the growth of the organism and (2) the
at the material may be air-dried rapidly and rendered relatively

*uction from Thin Stillage.* Beckord, Peltier, and Kneen (1946)
ported on the laboratory-scale production of amylases from thin
. The latter is obtained as a by-product of the ethyl alcohol fer-
ion of converted grains and contains water solubles derived from
lt-saccharified grains and the malt, grain fines, and nonviable
ells.

organism employed for amylase production by Beckord and his
tes was a strain of *B. subtilis* designated as isolate no. 23, which
en described by Beckord, Kneen, and Lewis. This bacillus charac-
ally produced a pellicle on thin stillage. Amylases were produced
bacteria making up the pellicle and secreted into the medium
below. Most of the enzymes were to be found in a rather narrow
mmediately below the pellicle unless the medium was agitated.

te 23 was cultured on peptone–beef-extract agar slants. A loopful
bacterial growth was used to inoculate a 50-ml. portion of sterile
llage (at a pH of 7 to 8) contained in a screw-capped glass bottle
by 14 cm.). After incubation at 37°C. for 24 hr. this culture was

used to inoculate the medium used for amylase production or to ino a second subculture.

Beckord and his associates produced amylases from thin stilla several methods: a shallow-pan method, an agitation method, and a method. In each case, the stillage was neutralized with sodium carb in such manner that the pH after sterilization was 7 to 8.

In the shallow-pan method, the sterilized thin stillage dispens layers 2.5 to 3.8 cm. deep was inoculated and incubated in an undistu condition for 6 days. It was found that amylase activity (based o dextrinization time in minutes at 30°C.) began to decrease after 6

In the agitation method, portions of thin stillage too deep for effi amylase production by the surface culture (quiescent) method were i ulated with the thin stillage culture of *B. subtilis* (isolate no. 23) and i bated at room temperature for 24 hr. Then a stirrer was put into opera which agitated the medium below the pellicle without disturbing pellicle. This, of course, brought fresh medium into contact with pellicle and removed metabolic products. A control, using the shallow- method, was carried out at the same time. This method was superio the quiescent method.

In the drip method, devised by Beckord and his collaborators, ste thin stillage was permitted to drip at a controlled rate down over a colu of moist poplar chips in a glass tube 60 mm. in diameter and 1.5 m. lo The top of the tube contained a rubber stopper, through which four in tubes conducted the stillage to the top of the column, and an air outl The bottom of the tube likewise contained a rubber stopper with an inlet tube and a stillage outlet tube. The apparatus was sterilized w steam at atmospheric pressure for 3 hr. before use. After cooling, t chips were inoculated with 500 ml. of a thin stillage culture grown 24 hr. at 30°C. The tube was incubated for 24 hr. at room temperat to encourage the development of the organisms on the chip surfaces. liters of sterilized wheat-corn stillage at a pH of 7 were allowed to d through the four inlet tubes and down over the chips at a controlled r of 3 liters per day. Sterile air was passed up through the tube. T amylase activity of the portion collected after the first day of flow w equal to that obtained in 5 days by the quiescent-medium method. T activity of the portion collected on the second day was of the same val However, repassage of these portions through the tube resulted in product of lower activity.

Attempts to produce amylase by submerged growth of the organi resulted in low activity even after a long period of incubation.

It was demonstrated that shaking the culture at daily intervals encourage the formation of new pellicles did not increase total amyl production. Replacement of the medium below a pellicle with fre medium at daily intervals did not increase the total amount of amyl produced, but it did increase the rate at which the maximum level w obtained.

*Qualitative and Quantitative Nature of Bacterial Amylases.* Kne and Beckord (1946) studied the quantity and quality of amylase pr

y 43 amylase-producing cultures of the *B. subtilis* group, 7 cultures
*olymyxa*, and 3 cultures of *B. macerans*. The medium used for the
of the cultures of *B. subtilis* was essentially a bran-extract–
e-phosphate medium which was prepared as follows: Bran and
l water were mixed in the ratio of 1 part of the former to 20 parts
latter (by weight), autoclaved at 15 lb. pressure for 20 min., and
l through cheesecloth. To each liter of the bran extract were
10 g. of Bacto peptone, 0.7 g. of $K_2HPO_4 \cdot 3H_2O$, and 0.3 g. of
$)_4$. After the constituents were dissolved, 200-ml. portions were
ed into glass culture containers (to produce a layer about 3 cm.
and autoclaved at 15 lb. pressure for 20 min. The medium used
growth of *B. polymyxa* and *B. macerans* was identical to the fore-
xcept that 20 g. per liter of $CaCO_3$ was added for the purpose of
izing the acids produced during the growth of these organisms. A
edium was prepared by adding 1.5 per cent of agar to the bran-
–peptone–phosphate broth.

inoculum for a 200-ml. portion of the above-described media was
d by growing the organism on a slant of the solid medium for
t 35°C. and suspending the growth in 5 ml. of sterile water.

bacteria were grown for 4 days at 35°C. for the production of the
m amylase content, according to Kneen and Beckord (1946).

uation of dextrinizing and saccharifying activities were carried
utlined on page 502.

*ification.* On the basis of their starch dextrinization and sacchari-
actions, Kneen and Beckord classified the amylase-producing
ms that they studied into four groups. These were as follows:

p 1. *B. subtilis* (saccharifying type). The production of amylase
rom little or none to relatively large amounts. During the period
h dextrinization, but little saccharification occurs; however, sac-
ation is pronounced during the postdextrinization period. The level
onversion of starch to sugar may be high. An inhibitor present in
etards the action of the amylase. The bacteria of this group may
ted from plant materials.

) 2. *B. subtilis* (nonsaccharifying or α-amylase type). Large
es of characteristic α-amylase are produced by organisms of this
The enzyme seems to be much like the commercial type of bac-
nylase. Bacteria producing this type of amylase may be isolated
py bread.

p 3. *B. polymyxa*. The amylase or amylase system possesses
degrading abilities like those of an extract of barley malt. The
fermentable sugar production is high during both the dextriniza-
d postdextrinization periods, and conversion comparable to that
d by malt is obtained.

) 4. *B. macerans*. The starch is converted initially to "Schar-
lextrins," which are nonreducing and nonfermentable, but there
a progressive production of fermentable sugars. High levels of
ion may be obtained as a result of using high enzyme concentra-
sufficient time.

*Evaluation of Dextrinizing Activity.* The dextrinizing activit time in minutes required by the enzyme to hydrolyze 20 ml. of 1 p gelatinized starch to the point where a red-brown color is produc iodine—was carried out by Kneen and Beckord, using a modifica the Wohlgemuth (1908) method. In carrying out the test, a 20-m tion of 1 per cent boiled soluble starch is buffered to a pH of 6. phosphates (3.0 g. of $KH_2PO_4$ and 0.6 g. of $K_2HPO_4 \cdot 3H_2O$ per lite their studies concerned with the production of amylases on wheat Beckord, Kneen, and Lewis added a 10-ml. aliquot of a 1:50 e (50 ml. of extractant per 1 g. of dry bran) of the bacterial bran 20 ml. of soluble starch. In their studies with liquid substrates, K and Beckord added 10 ml. of enzyme preparation to the soluble sta which 0.5 to 10 ml. was enzyme-containing substrate. The conv temperature was 30°C.

*Evaluation of Starch-saccharifying Activity.* The following proc was used by Kneen and Beckord for evaluating the saccharification a ties of amylase preparations: In brief, it consists of measuring the ca dioxide produced during the yeast fermentation of the hydrolytic pro resulting from the action of the amylase on soluble starch. The co sions, fermentations, and gas measurements are carried out in a "pre meter" (Sandstedt and Blish, 1934). Fifteen ml. of a solution that tains 0.6 g. of boiled soluble starch, 0.05 g. of asparagine, 0.25 g $(NH_4)_2SO_4$, 0.016 g. of NaCl, 0.04 g. of $MgSO_4 \cdot 7H_2O$, 0.04 g. of $KH_2$ 0.011 g. of $K_2HPO_4 \cdot 3H_2O$, and 0.03 g. of dextrose (used to accelerate fermentation) is added to each of the cups of the pressure meter. amylase-containing extract together with enough distilled water to m a total volume of 10 ml. are added to the 15 ml. of nutrient starch solu described above. The mixture is incubated at 30°C. for 1 hr. Then 5 of a suspension prepared to contain 0.5 g. of compressed bakers' ye 0.00008 g. of thiamin, and 0.00008 g. of pyridoxine are added, the to the pressure meter is affixed, and incubation is carried out at 30°C. pressure is equalized at the end of 5 min.

**Alpha-amylase.** The α-amylase produced by *B. subtilis* has an o mum pH of 6.5 to 8, is active at relatively high temperatures, 75 to 80 (even up to 95°C.), and may resist a short boiling in the presence starch when the pH of the medium is 7 to 8. α-Amylase is the m amylase of bacteria. This enzyme is dextrinogenic.

**Penicillinase.** This enzyme is produced by a number of microorg isms, including members of the *B. subtilis-mesentericus* group, *B. thracis,* some strains of *Micrococcus pyogenes* var. *aureus* and *Escheric coli,* and other bacteria (Florey et al., 1949). Its preparation from broth cultures of *Actinomyces candidus* (Welch, 1952) has been patent It is used to inactivate penicillin when the antibiotic is tested for sterili It inactivates penicillin by hydrolyzing the peptide linkage in the be lactam ring. It is also used in the making of Gruyère cheese (Aucl 1951). It destroys the inhibitory property that milk from cows trea with penicillin possesses for lactic acid starters. It has been used succe fully to combat the serious reactions which occasionally follow the inj penicillin into human beings (*Chemical and Engineering News*, 4. 1957).

**s of Bacterial Enzymes.** The amylases and proteases produced teria may be used for the same general purposes as those produced lds (see Chap. 41). Bacterial amylases may be used for the desiz- textiles; in the preparation of sizes for paper; for the production ch-conversion products of low fermentability, which are occasion- ed in the brewing industry; in the liquefaction of unmalted cereals; clarification of beer; in the preparation of cholocate sirups, in case the chocolate starch is dextrinized and thus the sirup does not e thick; and for other purposes.

erial proteases may be used for the unhairing and bating of hides; degumming of silk; for desizing acetate rayon when the size is f gelatin or casein; for the separation of silver from photographic y digestion of the gelatin of the film and liberation of the silver nd for other purposes.

## RIBOFLAVIN

production of riboflavin (vitamin G) by bacteria of the acetone- l group has been described by Yamasaki (1942), by Meade, Pol- nd Rodgers (1945), by Leviton (1946), and by others.

fly, the process involves the selection, preparation, and sterilization itable carbohydrate-containing mash; the addition of a buffer, such $CO_3$, prior to or during the fermentation; the inoculation of the with a pure culture of *Clostridium acetobutylicum* or related organ- nd incubation at 37 to 40°C. for 48 to 72 hr.

irly large number of raw materials may be used for the fermenta- Yamasaki, for example, mentions in his patent the use of mashes ed from barley, corn (maize), millet, oats, rye, and sorghum (these ning the cereals in 4 to 8 per cent concentrations); of a whey mash, yeast water mashes containing approximately 3 per cent of fer- le carbohydrate. Meade and associates, in their patent, are par- ly concerned with lactose-containing lacteal material, such as whey n milk. Leviton used a semisynthetic medium containing starch as mentable carbohydrate.

pH of the mash is adjusted to 6 to 7. If too acid at the beginning, sh may be neutralized with calcium, sodium, or potassium hydrox- he use of a buffer, such as $CaCO_3$, in a concentration of 0.1 to 0.3 nt is recommended (for example, 1 to 3 lb. of $CaCO_3$ per 1,000 lb. y). The sterile $CaCO_3$ may be added to the mash after it has been zed.

hey mash may be sterilized at a temperature of about 121°C. F.) (15 lb. steam pressure) for 15 to 20 min.; a starch-containing for 1 to 2 hr. at a pressure of 20 to 30 lb. per sq. in. (Yamasaki,

trol of the iron (ferrous) content of the mash is highly important production of riboflavin. The optimum concentration will vary

somewhat with the conditions of the fermentation but appears to be
1 to 3 parts per million. When the iron concentration is below or
the optimum range, riboflavin production is reduced.

Leviton has shown that riboflavin is decomposed rapidly by
solutions of hydrogen peroxide when in the presence of traces of f
ion. He also found that the iodide ion stabilized riboflavin towa
action of hydrogen peroxide in vitro and in vivo but that it was inhi
toward the microbiological synthesis of riboflavin.

The use of a suitable strain of *Cl. acetobutylicum,* such as A.T
4259 E. McCoy (strain T), is essential for highest yields of ribofla

The temperatures normally employed in the fermentation are
optimum for the acetone-butanol fermentation, namely, 37 to 4
depending upon the strain used.

Under the foregoing conditions, the yield of riboflavin in the ferme
tion medium may be materially increased. Yamasaki states that y
may be increased 30 to 50 times when cereal mashes are used and 1
more times when yeast water mashes are employed. Meade and cowor
report that yields by their process may be increased from about 1.4 to
to 50 $\gamma$ per ml. (240 to 2,200 $\gamma$ per g. on a dried basis).

## OXIDIZING ACTIVITIES OF STRAINS OF THE GENERA PSEUDOMONAS AND PHYTOMONAS[1]

The family Pseudomonadaceae contains two important genera, spe
of which possess oxidizing activities of value to mankind. These are
genera *Acetobacter* (refer to Chap. 23) and *Pseudomonas.* Some of
species described in the literature as members of the genus *Phytomona*
that possess oxidizing activities, also belong to this family.

**Oxidizing Activities of Pseudomonas Species.** Alsberg (19
observed the production of gluconic acid from a nutrient glucose solut
by *Ps. savastanoi.* Pervozvanskii (1939) reported that fluorescing bacte
produced gluconic and 2-ketogluconic acids in the presence of calci
carbonate during a fermentation period of 13 to 70 days. Pervozvans
and Ivashkevich (1939) reported that gluconic acid was the chief e
product formed by *Ps.* (*Bacterium*) *putidum.* The optimum conditio
for gluconic acid production were a temperature of 30°C., a glucose c
centration of 2 to 6 per cent, the presence of calcium carbonate, aerati
and periodic agitation. Stubbs, Lockwood, Roe, Tabenkin, and W
(1940) described the production of 2-ketogluconic acid in high yield fr
a nutrient glucose solution by an unnamed bacterium, which was la
identified as *Ps. fluorescens.* Later, Lockwood, Tabenkin, and W
(1941) demonstrated that several species of *Pseudomonas* were capable
producing 2-ketogluconic acid in "good yields." In 1946, Lockwood a
Stodola described the production of $\alpha$-ketogluconic acid by *Ps. fluoresc*
and Lockwood and Nelson reported on the production of pentonic ac
from *Pseudomonas* species. Stodola and Lockwood (1947) described t

---

[1] Refer to footnote *j* in Table 140.

140. THE PRODUCTION OF GLUCONIC ACID BY SPECIES OF *Pseudomonas* AND *Phytomonas*[a]

| Organism | Glucose[b] consumed, g./100 ml. | Reducing value of fermented liquors[c] | Optical rotation of fermented liquors[d] | Total calcium in solution, g./100 ml. | Gluconic acid formed: Equivalent to calcium in solution, g./100 ml. | Gluconic acid formed: Yield based on glucose consumed, per cent |
|---|---|---|---|---|---|---|
| *cidolens* 4685[e]... | 9.3 | 12 | + .68 | 0.81 | 7.9 | 78 |
| *cidolens* 4686[e]... | 8.4 | 16 | + .63 | 0.54 | 5.3 | 58 |
| *idolens* 4687[e]... | 8.4 | 29 | +1.03 | 0.90 | 8.8 | 96 |
| *xogenes* 946[e].... | 0.9 | 181 | +4.64 | 0 | 0 | 0 |
| *xogenes* 947[e].... | 1.4 | 167 | +4.18 | 0 | 0 | 0 |
| *ilis*[f] ........... | 10.0 | 7 | +1.02 | 1.02 | 10.0 | 92 |
| *um flavo-zona-* (*Xanth. niae*)[j] .......... | 1.9 | 165 | +4.54 | 0.11 | 1.1 | 52 |
| *campestris*[f,j] .. | 0.2 | 198 | ...... | 0 | 0 | 0 |
| *onafaciens*[h,j] ... | 0.9 | 179 | +4.33 | 0 | 0 | 0 |
| *bact. michi- nse*[f,j] ......... | 0.4 | 192 | +4.70 | 0 | 0 | 0 |
| *stewartii*[h,j] ..... | 2.3 | 157 | +4.33 | 0.10 | 1.0 | 40 |
| *afaciens*[h,j] ...... | 9.4 | 37 | + .70 | 0.84 | 8.2 | 80 |
| *ingae*[f,j] ........ | 7.2 | 51 | +1.98 | 0.65 | 6.4 | 82 |
| *ct. tume- is* A[i,j] ......... | 3.2 | 144 | +4.49 | 0 | 0 | 0 |
| *ct. tume- is* B[i,j] ......... | 4.1 | 124 | +4.95 | 0 | 0 | 0 |
| *ct. tume- is* C[i,j] ......... | 2.0 | 150 | +4.50 | 0 | 0 | 0 |

---

: Two hundred mm. of nutrient solution in gas-washing bottles contained 20 g.
ose. 5 g. of $CaCO_3$, 1 g. of corn-steep liquor, 0.4 g. of urea, 0.12 g. of $KH_2PO_4$,
of $MgSO_4 \cdot 7H_2O$, and 1 drop of oleic acid (antifoam agent). Temperature
ir flow 200 ml. per min., duration 8 days.

3. Lockwood, B. Tabenkin, and G. E. Ward, *Jour. Bact.*, 42(1): 51 (1941).

ginal concentration 9.3–10.0 per cent.

ressed in milligram of copper per milliliter of solution.

gree of optical rotation of sodium light at 20–25° for a 1-dm. tube.

eived from the American Type Culture Collection. Culture numbers are theirs.

eived from N. R. Smith, Bureau of Plant Industry, U.S. Department of Agri-
.

eived from Lucia McCulloch, Bureau of Plant Industry, U.S. Department of
ture.

eived from Charlotte Elliot, Bureau of Plant Industry, U.S. Department of
ture.

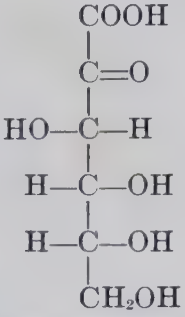
eived from A. J. Riker, University of Wisconsin.

se bacteria were originally listed as *Phytomonas* species by the authors, but
een reclassified as indicated in the seventh edition of Bergey's Manual (1957).

oxidation of lactose and maltose to bionic acids by a strain *graveolens.*

*Gluconic Acid Production.* A number of species of the genera *domonas* and *Xanthomonas* produce gluconic acid in quantity whe vated submerged in aerated nutrient-glucose media. However, n the strains of *Pseudomonas* oxidize the gluconic acid produced by t 2-ketogluconic acid. Lockwood, Tabenkin, and Ward (1941) foun one strain of *Ps. ovalis* formed gluconic acid only. Table 140 pr data concerning gluconic acid production by species of *Pseudomon* *Xanthomonas* and other bacteria.

*Production of* 2-*Ketogluconic Acid.* The structural formula of D-2 gluconic acid is as follows:



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{C{=}O} \\ | \\ \mathrm{HO{-}C{-}H} \\ | \\ \mathrm{H{-}C{-}OH} \\ | \\ \mathrm{H{-}C{-}OH} \\ | \\ \mathrm{CH_2OH} \end{array}$$

D-2-Ketogluconic acid

Lockwood, Tabenkin, and Ward (1941) found that 16 strains o species of the genus *Pseudomonas* produced 2-ketogluconic acid, the y being larger than 80 per cent in several cases as may be ascertained reference to Table 141.

A method for producing 2-ketogluconic acid in approximately 82 cent yields from 10 per cent glucose solutions in 25 hr. with an orga identified later as a strain of *Ps. fluorescens* has been described by Stu Lockwood, Roe, Tabenkin, and Ward (1940). The method employed essentially that described in Chap. 29 on page 581. There were s differences, however, which will be discussed now. The organism grown in a culture tube containing a 10-ml. portion of a medium cons ing of 5 per cent of glucose and 0.5 per cent of yeast extract. This used to inoculate 200 ml. of a medium of the same composition as sh on page 463. The nutrient solution used for the production of 2-keto conic acid contained the following ingredients in the amounts indicate

| Components | Amounts |
|---|---|
| Commercial glucose | 118 g.* |
| Corn-steep liquor | 5 g. |
| Octadecyl alcohol (antifoam agent) | 0.3 g. |
| Urea (sterilized separately) | 2 g. |
| $MgSO_4 \cdot 7H_2O$ | 0.25 g. |
| $KH_2PO_4$ | 0.60 g. |
| $CaCO_3$ (sterilized separately) | 27 g. |
| Distilled water to make | 1,000 ml. |

* Amount necessary for a 10 per cent glucose concentration.

| Organism | Glucose $^{a}$ consumed, g./100 ml. $^{b}$ | Reducing value of fermented liquor $^{c}$ | Optical rotation of fermented liquor $^{d}$ | acid formed |  |  |  |  |
|---|---|---|---|---|---|---|---|---|
|  |  |  |  | Found, g./100 ml. of solution | Yield based on glucose consumed, per cent | Total in solution, g./100 ml. | Due to 2-keto-gluconic acid |  |
|  |  |  |  |  |  |  | G./100 ml. | Per cent of total |
| *Ps. aeruginosa* $^{e}$ | 8.1 | 114 | −4.90 | 5.8 | 67 | 0.62 | 0.60 | 97 |
| *Ps. aeruginosa* $^{e}$ | 7.8 | 124 | −4.90 | 6.0 | 71 | 0.64 | 0.62 | 97 |
| *Ps. fluorescens* $^{f}$ | 9.3 | 67 | −2.29 | 3.0 | 30 | 0.97 | 0.31 | 32 |
| *Ps. fluorescens* $^{e}$ | 8.7 | 164 | −6.05 | 7.7 | 82 | 0.84 | 0.79 | 94 |
| *Ps. fluorescens* 948 $^{g}$ | 8.3 | 166 | −6.32 | 7.9 | 88 | 0.84 | 0.82 | 97 |
| *Ps. fluorescens* 949 $^{g}$ | 8.3 | 161 | −5.93 | 7.5 | 84 | 0.98 | 0.77 | 79 |
| *Ps. fluorescens* 142 $^{g}$ | 5.8 | 166 | −2.14 | 4.7 | 75 | 0.52 | 0.49 | 93 |
| *Ps. fragi* 4973 $^{g}$ | 9.7 | 165 | −7.77 | 9.0 | 86 | 1.03 | 0.93 | 90 |
| *Ps. graveolens* 4683 $^{g}$ | 9.7 | 155 | −6.82 | 8.0 | 77 | 0.93 | 0.83 | 89 |
| *Ps. graveolens* 4684 $^{g}$ | 7.5 | 166 | −4.60 | 6.6 | 82 | 0.69 | 0.68 | 98 |
| *Ps. mildenbergii* 795 $^{g}$ | 9.7 | 186 | −9.11 | 10.5 | 100 | 1.08 | 1.08 | 100 |
| *Ps. ovalis* 950 $^{g}$ | 7.6 | 117 | −3.09 | 4.5 | 55 | 0.63 | 0.46 | 73 |
| *Ps. pavonacea* 951 $^{g}$ | 5.5 | 170 | −1.77 | 4.6 | 77 | 0.58 | 0.48 | 82 |
| *Ps. putida* 4359 $^{g}$ | 8.7 | 162 | −6.62 | 8.0 | 85 | 0.92 | 0.83 | 90 |
| *Ps. schuylkilliensis* $^{e}$ | 8.6 | 166 | −6.00 | 7.6 | 82 | 0.91 | 0.78 | 86 |
| *Ps. vendrelli* 7700 $^{g}$ | 7.1 | 168 | −4.06 | 6.2 | 81 | 0.65 | 0.64 | 98 |

NOTE: Two hundred ml. of nutrient solution in gas-washing bottles contained 20 g. of glucose, 5 g. of $CaCO_3$, 1 g. of corn-steep liquor, 0.4 g. of urea, 0.12 g. of $KH_2PO_4$, 0.05 g. of $MgSO_4 \cdot 7H_2O$, and 1 drop of oleic acid (antifoam agent). Temperature 30°C., air flow 200 ml. per min. Duration 8 days.

[a] L. B. Lockwood, B. T. Tabenkin, and G. E. Ward, *Jour. Bact.*, **42**(1): 51 (1941).

[b] Original concentration 9.6 to 10 per cent.

[c] Expressed in milligrams of copper per milliliter of solution.

[d] Degrees of rotation at 20 to 25° in a 1-dm. tube.

[e] Received from N. R. Smith, Bureau of Plant Industry, U. S. Department of Agriculture.

[f] Our isolation.

[g] Received from the American Type Culture Collection. Culture numbers are theirs.

The sterilized medium in the amount of 3 liters was inoculat 300 ml. of inoculum and placed in a rotary drum. The condi fermentation were identical to those described on page 463.

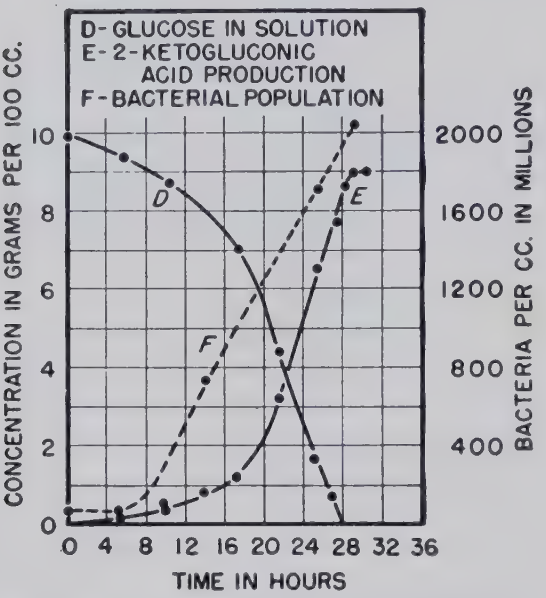


FIG. 86. Course of typical 2-ketogluconic acid fermentation. [*Courtesy of J. J. Stubbs, L. B. Lockwood, E. T. Roe, B. Tabenkin, and G. E. Ward, Ind. Eng. Chem.,* 32: 1626 (1940). *Copyright* 1940 *by the American Chemical Society and reprinted by permission of the copyright owner.*]

The course of the 2-ketogluconic acid fermentation is indic Fig. 86. According to Stubbs and his associates, the formation acid occurs simultaneousl glucose utilization and the b grow during the course of t mentation.

*Production of Pentonic* Lockwood and Nelson (1946 described the production of bonic acid, L-arabonic acid, lonic acid, and D-ribonic aci D-arabinose, L-arabinose, D-x and D-ribose, respectively. acids were produced in 250-m tubes, which were equipped aerator stones and which cont 100 ml. of the following medium: 2 g. of urea, 0.6 $KH_2PO_4$, 0.25 g. of $MgSO_4 \cdot 7$ and 5 ml. of corn-steep liqu liter. The media were steriliz passage through Seitz steri pads, inoculated with the de species of *Pseudomonas*, and bated at 30°C. Each tube was ated with 100 ml. per min. of f sterilized air. Excessive frothing of the cultures was prevented by ac 3 drops of soybean oil to each tube. Enough sterilized calcium carb was added to each tube at the time of inoculation to neutralize the that was expected to be formed.

The findings of Lockwood and Nelson are summarized in Table 14

*Production of Bionic Acids.* The production of bionic acids by oxidation of lactose and maltose, respectively, has been describe Stodola and Lockwood (1947).

*Ps. graveolens* proved to be the best of 15 species of *Pseudom* studied for the production of calcium lactobionate from a nutrient la solution containing calcium carbonate. It was also the best specie of 18 examined for the production of calcium maltobionate under si conditions. Yields of 75 per cent of calcium lactobionate and 77 per of calcium maltobionate were obtained in rotating drums (refer to 580) in 165 hr. and 50 hr., respectively. Other species of *Pseudom* were found to oxidize lactose and maltose, but their rate of oxid was too slow to be of interest.

Inocula were prepared as follows: A 24-hr. culture of *Ps. grave* (or other species), grown in 8 ml. of a liver-extract medium conta

142. PENTONIC ACIDS AND *Pseudomonas* SPECIES WHICH PRODUCE THEM *

| onic acid † | L-Arabonic acid ‡ | D-Xylonic acid § | D-Ribonic acid ¶ |
|---|---|---|---|
| agi | *Ps. fluorescens* | *Ps. fluorescens* | *Ps. fluorescens* |
| aveolens | *Ps. fragi* | *Ps. fragi* | *Ps. fragi* |
| nxantha | *Ps. mildenbergii* | *Ps. graveolens* | *Ps. graveolens* |
| ndrelli | *Ps. putida* | *Ps. mildenbergii* | *Ps. mildenbergii* |
| | *Ps. synxantha* | *Ps. ovalis* | *Ps. ovalis* |
| | *Ps. vendrelli* | *Ps. putida* | *Ps. putrifaciens* |
| | | | *Ps. synxantha* |
| | | | *Ps. vendrelli* |

pared from data presented in article by L. B. Lockwood and G. E. N. Nelson, *act.*, 52(5): 581 (1946).

lium contained 4.8 g. of D-arabinose per 100 ml.

lium contained 4.9 g. of L-arabinose per 100 ml.

lium contained 6.7 g. of D-xylose per 100 ml.

lium contained 5.9 g. of D-ribose per 100 ml.

r cent of glucose, was used to inoculate 100 ml. of fermentation n in a Pyrex tube fitted with finely porous stones for aeration es. Each tube also received 1 ml. of a sterile (20 per cent) urea n and 2.5 g. of sterilized, dry $CaCO_3$ at this time. The tubes were d with 100 ml. of air per minute while being incubated at 30°C.

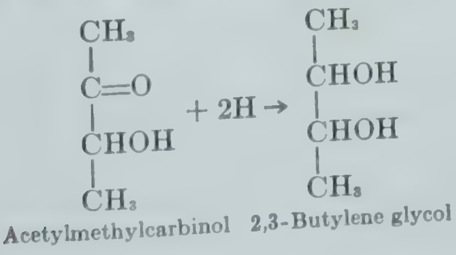
fermentation medium contained per liter about 100 g. of the sugar, of $KH_2PO_4$, 0.25 g. of $MgSO_4 \cdot 7H_2O$, and 5 ml. of corn-steep liquor. arrying out fermentations with the rotary fermentors, 100 ml. of im, prepared as described above, was used to seed 3,000 ml. of the fermentation medium in a drum. During the fermentation, the eceived 1,200 ml. of air per minute, while being rotated at the rate r.p.m. The temperature was maintained at 25°C.

he completion of fermentation, 100-ml. portions of the culture were filtered and dried by lyophilization and analyzed.

## ACETYLMETHYLCARBINOL, 2,3-BUTYLENE GLYCOL, AND DIACETYL

ylmethylcarbinol and 2,3-butylene glycol are frequently found er in certain fermentations. Refer to Chap. 22 for a consideration 2,3-butylene glycol fermentation.

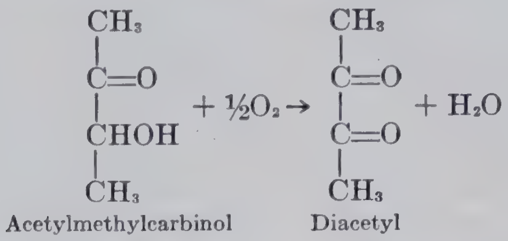
ie bacteria have the ability to reduce acetylmethylcarbinol to tylene glycol, a change that may be indicated as follows:



$$\begin{array}{c} CH_3 \\ | \\ C{=}O \\ | \\ CHOH \\ | \\ CH_3 \end{array} + 2H \rightarrow \begin{array}{c} CH_3 \\ | \\ CHOH \\ | \\ CHOH \\ | \\ CH_3 \end{array}$$

Acetylmethylcarbinol 2,3-Butylene glycol

Other bacteria are capable of oxidizing 2,3-butylene glycol to
methylcarbinol. One such organism is *Aerobacter aerogenes.* Stil
bacteria may oxidize acetylmethylcarbinol to diacetyl:


$$CH_3COCHOHCH_3 + \tfrac{1}{2}O_2 \rightarrow CH_3COCOCH_3 + H_2O$$

Diacetyl is important as an aroma-producing component of b
Butter cultures of the desired type always contain diacetyl and ac
methylcarbinol in relatively large amounts. The latter substance i
dized to diacetyl in butter cultures by such citric acid fermenting or
isms as *Leuconostoc dextranicum* and *L. citrovorum,* according to Han
and Babel (1957). The production of acetylmethylcarbinol and e
cially diacetyl by butter cultures or pure cultures of the citric-a
fermenting streptococci is favored by the acidification of the cultures
a mixture of citric and sulfuric acids and the presence of an abund
supply of oxygen. Obviously the reductions of diacetyl and acetylmet
carbinol to 2,3-butylene glycol cause a diminution in the amount of
aroma-producing substance, diacetyl.

## FERMENTATION OF RHAMNOSE BY BACTERIUM RHAMNOSIFERMENTANS

The fermentation of rhamnose ($CH_3\cdot CHOH\cdot CHOH\cdot CHOH\cdot CHO$
CHO) by *Bacterium rhamnosifermentans* is of particular interest, beca

TABLE 143. QUANTITATIVE RESULTS OF THE FERMENTATION OF RHAMNOSE BY *Bacter*
*rhamnosifermentans* *

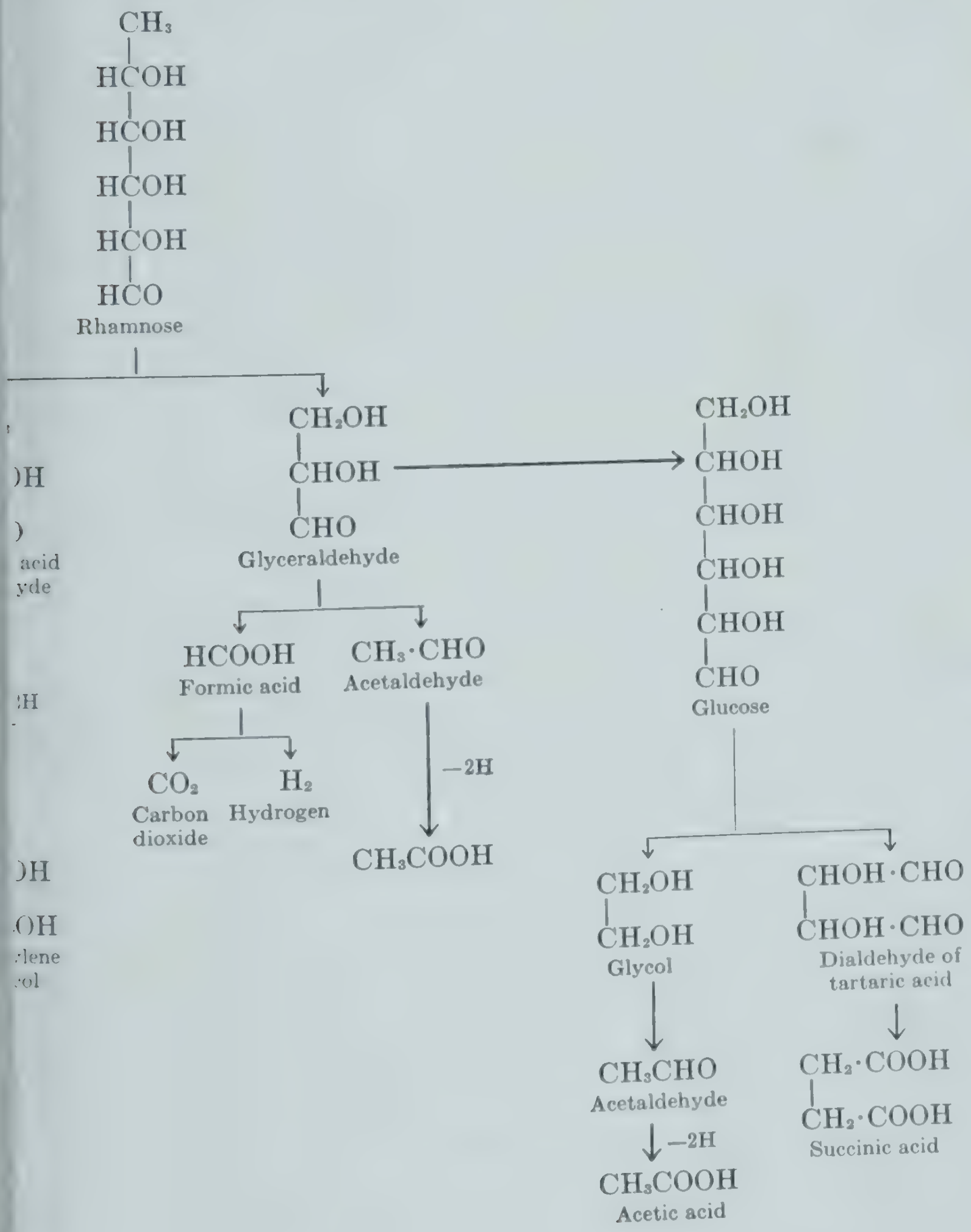
| | Weight, g. | Per cent carbon | mM. | mM./100 mM. fermented rhamnose | Available hydrogen, meq. | Availa hydrog per ce |
|---|---|---|---|---|---|---|
| Rhamnose: | | | | | | |
| Added ........ | 9.956 | | | | | |
| Recovered .... | 2.737 | | | | | |
| Fermented .... | 7.210 | 100 | 44 | 100 | 2,600 | 100 |
| Carbon dioxide .. | 0.623 | 5.36 | 14 | 31.8 | | |
| Hydrogen ...... | 0.032 | ..... | 14 | 31.8 | 63.6 | 2.4 |
| Formic acid ..... | 0.057 | 0.50 | 1 | 2.3 | 4.6 | 0.2 |
| Acetic acid ..... | 1.935 | 24.42 | 32 | 73.0 | 584.0 | 22.5 |
| Ethanol ........ | 0.056 | 1.00 | 1 | 2.3 | 27.6 | 1.1 |
| Succinic acid .... | 1.752 | 22.50 | 15 | 34.0 | 476.0 | 18.3 |
| Propylene glycol.. | 3.344 † | 50.00 | 44 | 100 | 1,600 | 61.0 |
| Total ....... | .... | 103.78 | .. | ..... | ..... | 105.5 |

* A. J. Kluyver and Ch. Schnellen, *Enzymologia,* **4**: 7–12 (1937).
† Not determined, but computed.

ene glycol ($CH_3 \cdot CHOH \cdot CH_2OH$) is formed as one of the end
ts (Kluyver and Schnellen, 1937).

organism was described by Castellani (1931) as *Bacillus rhamnosi-*
*itans*. It is a Gram-negative, facultative aerobe, which shows selec-
tion in respect to the carbohydrates fermented and the nitrogen
unds utilized. Rhamnose in peptone water (1 per cent) containing
r cent of calcium carbonate was fermented nearly completely in
s by *Bact. rhamnosifermentans*, and more vigorously than any other
ydrate investigated. Yeast water was unfavorable as a nitrogen
. Equimolar quantities of hydrogen and carbon dioxide gases were
ed by *Bact. rhamnosifermentans*, which is believed to be closely
l to *Escherichia coli*.

le 143 shows the products identified and quantitatively estimated
fermentation of a medium, which contained 1.5 per cent rhamnose


CH₃
HCOH
HCOH
HCOH
HCOH
HCO
Rhamnose
)H
)
acid
yde
CH₂OH
CHOH
CHO
Glyceraldehyde
CH₂OH
CHOH
CHOH
CHOH
CHOH
CHO
Glucose
HCOOH
Formic acid
CH₃·CHO
Acetaldehyde
:H
−2H
CO₂
Carbon dioxide
H₂
Hydrogen
CH₃COOH
)H
OH
lene
ol
CH₂OH
CH₂OH
Glycol
CHOH·CHO
CHOH·CHO
Dialdehyde of tartaric acid
CH₃CHO
Acetaldehyde
−2H
CH₃COOH
Acetic acid
CH₂·COOH
CH₂·COOH
Succinic acid

and 1.5 per cent calcium carbonate in peptone water. The amoun propylene glycol represented was obtained by calculation and not quantitative estimation of its content in the fermentation medium.

On the basis of the available facts and the assumption concern propylene glycol, Kluyver and Schnellen have suggested the forego scheme to indicate the course of the fermentation of rhamnose by *B rhamnosifermentans.*

## Some References on Minor Fermentations

Barker, H. A.: On the Biochemistry of the Methane Fermentation, *Arch. Mikrob* **7**: 404–419 (1936).

———: The Production of Caproic and Butyric Acids by the Methane Fermenta of Ethyl Alcohol, *Arch. Mikrobiol.,* **8**: 415–421 (1937).

———: On the Fermentation of Glutamic Acid, *Enzymologia,* **2**: 175–182 (1937).

Bois, E., and J. Savary: *Can. Jour. Research* **B, 23**: 208 (1945).

Butlin, K. R.: "The Biochemical Activities of the Acetic Acid Bacteria," Chemist Research, Special Report 2, H.M. Stationery Office, London, 1936.

Castellani, A.: Sur un bacille déterminant la fermentation du rhamnose avec dégag ment de gaz, à l'exclusion de tout autre hydrate de carbone, glucoside, alco ou composé hydro-aromatique, d'une série de 28 de ces substances exam nées. Emploi de ce bacille pour découvrir et identifier le rhamnose, *Ann. ins Pasteur,* **47**: 297–305 (1931).

Donker, H. J. L.: Bijdrage tot de kennis der boterzuurbutylalcoholen acetongi tingen, thesis, Eidgenössischen Technische Hoogeschool, Delft, 1926.

Evans, T. H., and H. Hibbert: Bacterial Polysaccharides, *Scientific Report Series N* 6, Sugar Research Foundation, Inc., New York, April, 1947.

Giovannozzi, M.: *Chimica e industria* (*Milan*), **40**: 768: 1937.

Hockenhull, D. J. D., and D. Herbert: *Biochem. Jour.,* **39**: 102 (1945).

Janke, A., and B. Schaefer, *Zentr. Bakt. Parasitenk.,* II, **102**: 241 (1940).

Kluyver, A. J.: "The Chemical Activities of Micro-organisms," University of Londo Press, Ltd., London, 1931.

———: Microbial Metabolism and Its Significance to the Microbiologist, *Intern Congr. Microbiol., Rept. Proc., 3d Congr., New York,* 1940.

——— und Ch. Schnellen: Über die Vergärung von Rhamnose, *Enzymologia,* **4**: 7-1 (1937).

——— and ———: On the Fermentation of Carbon Monoxide by Pure Cultures o Methane Bacteria, *Arch. Biochem.,* **14**(1 and 2): 57–70 (1947).

Kneen, E., and R. M. Sandstedt: *Arch. Biochem.,* **9**: 235 (1946).

Langlykke, A. J., W. H. Peterson, and E. McCoy: Products from the Fermentatio of Glucose and Arabinose by Butyric Acid Anaerobes, *Jour. Bact.,* **29**: 333 (1935

Lindner, P.: "Mikroskopische und biologische Betriebskontrolle in den Gärungs gewerben," 6th ed., Paul Parey, Berlin, 1930, p. 585.

Peltier, G. L., and L. D. Beckord: *Jour. Bact.,* **50**: 711 (1945).

Schultz, A., L. Atkin, and C. N. Frey: U.S. Patent 2,159,678, May 23, 1939.

Smit, J.: "Die Gärungssarcinen," Gustav Fischer Verlagsbuchhandlung, Jena, Ger many, 1930.

Waldmann, R.: U.S. Patent 2,302,079, Nov. 17, 1942.

Wallerstein, L.: Enzyme Preparations from Microorganisms, Commercial Productio and Industrial Application, *Ind. Eng. Chem.,* **31**: 1218 (1939).

Weizmann, C.: Preliminary Note on an Ethyl Alcohol Producing Bacillus Isolate from *Hibiscus sabdariffa* L. (Rozella), *Jour. Soc. Chem. Ind.,* **57**: 1049 (1938).

Willaman, J. J.: Industrial Use of Microbial Enzymes, *Abstr. Communs., Inter Congr. Microbiol., 3d Congr., New York,* Sept. 2–9, 1939, pp. 335–336.

Wohlgemuth, J.: *Biochem. Zeit.,* **9**: 1 (1908).

## Some References on Bacterial Enzymes

der, M., and P. W. Wilson: Large-scale Production of *Azotobacter* for
zymes, *Appl. Microbiol.*, **2**: 135–140 (1954).

, J.: Inhibitory Action of Penicillin on the Lactobacilli Used in the Manufac-
e of Gruyère Cheese, *Lait*, **31**: 121–133 (1951).

ood, A. C.: Production of Cytases Active on Barley Gum by Bacteria of the
nus *Bacillus*, *Can. Jour. Botany*, **31**: 28–32 (1953).

H. W., et al.: "Antibiotics," Oxford University Press, New York, 1949, Vol. II,
. 1090–1100.

W. B.: The Preparation of Cell-free Enzymes from Microorganisms, *Bact.*
*rs.*, **18**(2): 87–105 (1954). (This article contains 126 references to the lit-
ture.)

ood, L .B.: *Trans. N.Y. Acad. Sci.*, Ser. II, **15**: 1–5 (1952).

mer, S., and J. A. Garibaldi: Further Studies on the Production, Purification
l Properties of the Schardinger Dextrinogenase of *Bacillus macerans*, *Cereal*
*em.*, **29**(2): 108–122 (1952).

H.: Hydrolysis of Beta-Lactam Linkage by Penicillinase, U.S. Patent 2,601,-
), June 24, 1952.

## Some References concerning Bacterial Amylases

d, L. D., E. Kneen, and K. H. Lewis: Bacterial Amylases, Production on Wheat
an, *Ind. Eng. Chem.*, **37**: 692–696 (1945).

G. L. Peltier, and E. Kneen: Bacterial Amylases Production in Thin Stillage,
*l. Eng. Chem.*, **38**: 232–238 (1946).

M. J., and R. M. Sandstedt: Biocatalytic Activators Specific for the Yeast
rmentation of Maltose, *Jour. Biol. Chem.*, **118**: 765–780 (1937).

lo, F. J., S. Redfern: α-Amylase from *Bacillus subtilis:* I. Purification and
ysical Properties, *Arch. Biochem.*, **15**(3): 333–342 (1947).

and ———: α-Amylase from *Bacillus subtilis:* II. Essential Groups, *Arch.*
*ochem.*, **15**(3): 343–350 (1947).

and ———: α-Amylase from *Bacillus subtilis:* III. Effects of Inhibitors upon
uefaction and Dextrinization, *Arch. Biochem.*, **17**(1): 1–2 (1948).

. C., and J. A. Jump: Microbial Amylase Preparations, *Ind. Eng. Chem.*, **37**:
–525 (1945).

s, R. H., and D. Kulka: Some Comparisons between a Bacterial Amylase and
Malt Amylase. I. The Kinetics of Starch Saccharification by the Two En-
nes, *Jour. Inst. Brewing*, **48**: 170–174 (1942).

L., L. R. MacDonnell, and H. Lineweaver: Bacterial Proteinase from Waste
paragus Butts, *Ind. Eng. Chem.*, **36**: 1152–1158 (1944).

E., and L. D. Beckord: Quantity and Quality of Amylose Produced by Vari-
Bacterial Isolates, *Arch. Biochem.*, **10**(1): 41–54 (1946).

R. M. Sandstedt, and C. M. Hollenbeck: The Differential Stability of the
lt Amylases—Separation of the Alpha and Beta Components, *Cereal Chem.*,
: 399–423 (1943).

edt, R. M., E. Kneen, and M. J. Blish: A Standardized Wohlgemuth Proce-
re for Alpha-amylase Activity, *Cereal Chem.*, **16**: 712–723 (1939).

. E. B., and C. S. Hudson: Conversion of Starch to Crystalline Dextrins by
Action of a New Type of Amylase Separated from Cultures of *Aerobacillus*
*cerans*, *Jour. Am. Chem. Soc.*, **61**: 2900–2902 (1939).

and ———: Preparation and Properties of the Amylases Produced by *Bacillus*
*cerans* and *Bacillus polymyxa*, *Jour. Bact.*, **43**: 527 (1942).

## Some References on Riboflavin

. A.: The Microbiological Synthesis of Riboflavin—A Theory concerning Its
ibition, *Jour. Am. Chem. Soc.*, **68**: 835–840 (1946).

——— and H. B. Haller: The Relation between Riboflavin and Hydrogen P in the Presence of Ferrous Ion, paper presented at 110th Meeting of the can Chemical Society, Chicago, Sept. 9–13, 1946.

Meade, R. E., H. L. Pollard, and N. E. Rodgers: Process for Manufacturing min Concentrate, U.S. Patent 2,369,680, Feb. 20, 1945.

Yamasaki, I.: Process of Preparing Growth Promoting Vitamin $B_2$ (Vitamin Fermentation, U.S. Patent 2,297,671, Sept. 29, 1942.

## Some References on the Genera Pseudomonas and Phytomonas

Alsberg, C. L.: The Formation of *d*-Gluconic Acid by *Bacterium savastanoi* *Jour. Biol. Chem.*, **9**: 1–7 (1911).

Lockwood, L. B., B. Tabenkin, and G. E. Ward: The Production of Gluconi and 2-Ketogluconic Acid from Glucose by Species of *Pseudomonas* and *P monas, Jour. Bact.*, **42**(1): 51–61 (1941).

——— and G. E. N. Nelson: The Oxidation of Pentoses by Pseudomonas, *Bact.*, **52**(5): 581–586 (1946).

——— and F. H. Stodola: Preliminary Studies on the Production of α-Ketogl Acid by *Pseudomonas fluorescens, Jour. Biol. Chem.*, **164**(1): 81–83 (1946).

——— and ———: α-Ketoglutaric Acid by Fermentation, U.S. Patent 2,443,919, 22, 1948.

Pervozvanskii, V. V.: Formation of Gluconic Acid during the Oxidation of Gl by Bacteria, *Mikrobiologiia,* **8**(2): 149–159 (1939); *Chem. Abstr.,* **34**: (1940).

——— and M. A. Ivashkevich: Formation of Gluconic Acids in *Bacterium puti* L. et N., *Mikrobiologiia,* **8**(3–4): 339–352 (1939); *Chem. Abstr.,* **34**: (1940).

Stanier, R. Y.: Acetic Acid Production from Ethanol by Fluorescent Pseudomon *Jour. Bact.,* **54**: 191–194 (1947).

Stodola, F. H., and L. B. Lockwood: The Oxidation of Lactose and Maltose Bionic Acids by Pseudomonas, *Jour. Biol. Chem.,* **171**: 213–221 (1947).

Stubbs, J. J., L. B. Lockwood, E. T. Roe, B. Tabenkin, and G. E. Ward: Bacte Production of Ketogluconic Acids from Glucose, *Ind. Eng. Chem.,* **32**: 1626–1 (1940).

Vaughn, R. H.: The Acetic Acid Bacteria, *Wallerstein Labs. Communs.,* **5**(14): 5 (1942).

## CHAPTER 27

# THE MOLDS

molds are members of the division of the plant kingdom known
llophyta. They do not possess chlorophyll nor are they differenti-
ıto leaves, stems, or true roots. They are widely distributed, espe-
n the soil.

role of molds in nature is of very great importance to man. Patho-
molds are the causes of diseases in plants and animals. Non-
enic species include those involved in the breakdown of organic
in the soil; those concerned in the deterioration and destruction
ber, textiles, foods, and other products; and those with distinct
ial importance, as the molds concerned with the ripening of cheese
e production of commercially valuable organic acids, enzyme prep-
ıs, sauces, and related products.

molds may be divided into four main classes: the Phycomycetes,
usually possess nonseptate mycelia; the Basidiomycetes, which
septate mycelia and produce sexual spores exogenously on stalks;
omycetes, which possess septate mycelia and produce sexual spores
nously in sacs; and the Fungi Imperfecti, which possess septate
ı but often produce no sexual spores, so far as is now known. (Refer
87 for subdivisions of these classes.)

**ral Description.** The individual mold, structurally, may be con-
to be made up principally of mycelium and spores. The mycelium
lection or aggregate of hyphae (singular, hypha), which are thread-
aments of protoplasm. Hyphae are of two main types: fertile
, which are concerned with the production of reproductive cells
bodies, the spores; and vegetative hyphae, the function of which
cure nutrient substances from the substrates.

hae may be septate or nonseptate. Septate hyphae are hyphae
ıing crosswalls, or septa, which divide the mold into cells. Non-
hyphae contain no crosswalls, but are multinucleate. Molds of
ter type are coenocytic. Cells of septate molds may contain only
cleus, as in the case of the Ascomycetes, or two nuclei, as in the
omycetes.

ls increase in mass or grow by the extension of the tip cells (apical
) or in septate types by apical growth and by a division of the cells
part of the hypha (intercalary growth).

young cells of a mold are usually filled with dense cytoplasm, but
s contain many vacuoles and reserve food materials, such as fat
s and glycogen. The cell wall is believed to be composed of chitin.

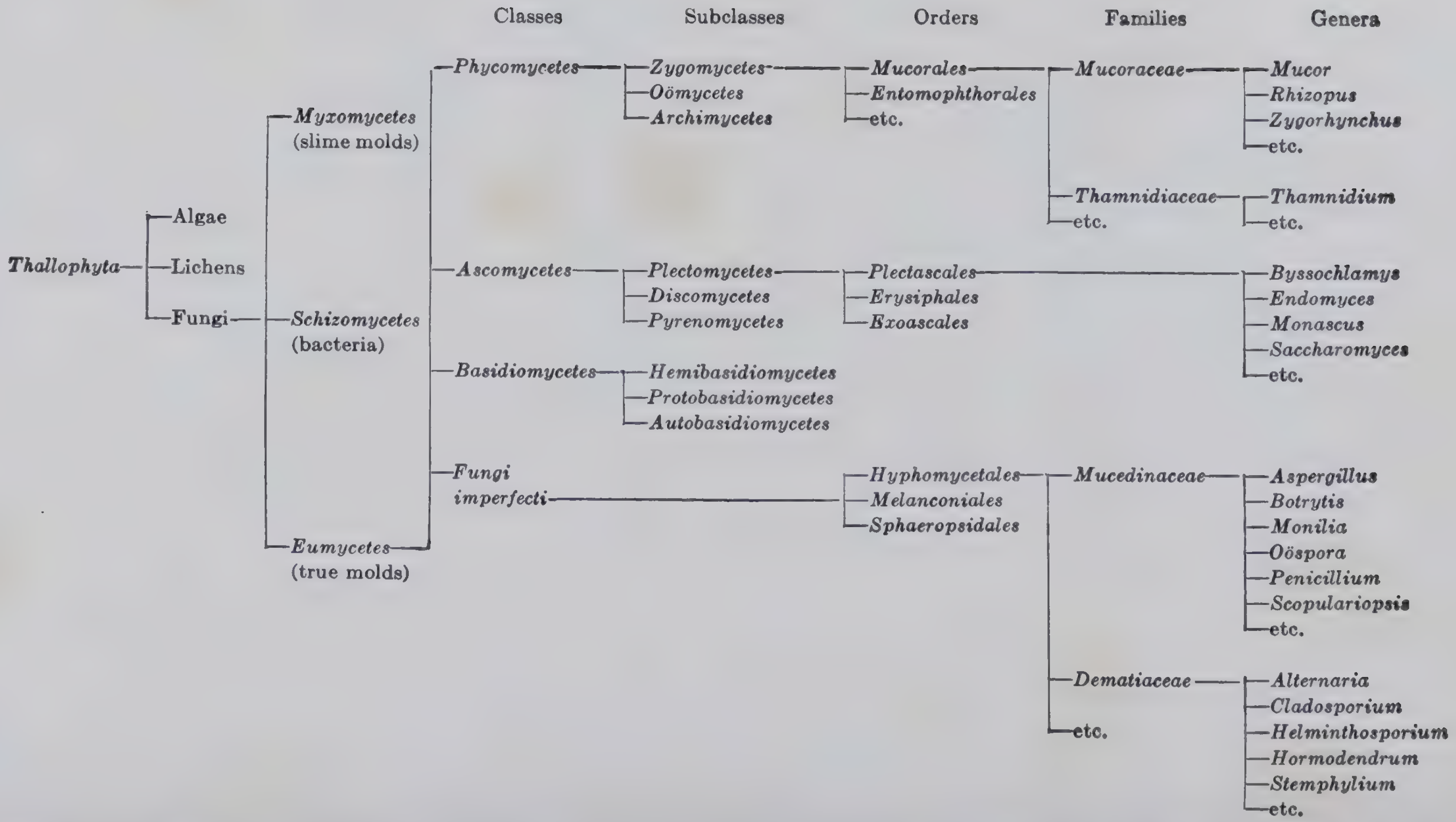

Classes
Subclasses
Orders
Families
Genera
Thallophyta
Algae
Lichens
Fungi
Myxomycetes (slime molds)
Schizomycetes (bacteria)
Eumycetes (true molds)
Phycomycetes
Ascomycetes
Basidiomycetes
Fungi imperfecti
Zygomycetes
Oömycetes
Archimycetes
Plectomycetes
Discomycetes
Pyrenomycetes
Hemibasidiomycetes
Protobasidiomycetes
Autobasidiomycetes
Mucorales
Entomophthorales
etc.
Plectascales
Erysiphales
Exoascales
Hyphomycetales
Melanconiales
Sphaeropsidales
Mucoraceae
Thamnidiaceae
etc.
Mucedinaceae
Dematiaceae
etc.
Mucor
Rhizopus
Zygorhynchus
etc.
Thamnidium
etc.
Byssochlamys
Endomyces
Monascus
Saccharomyces
etc.
Aspergillus
Botrytis
Monilia
Oöspora
Penicillium
Scopulariopsis
etc.
Alternaria
Cladosporium
Helminthosporium
Hormodendrum
Stemphylium
etc.

FIG. 87. A classification of the fungi. (*Courtesy of A. T. Henrici, "Molds, Yeasts, and Actinomycetes," John Wiley & Sons, Inc., New York*, 1930; *G. Smith, "An Introduction to Industrial Mycology," Edward Arnold & Co., London*, 1938; *H. M. Fitzpatrick, "The Lower Fungi—Phycomycetes," McGraw-Hill Book Company, Inc., New York*, 1930.)

res may be asexual or sexual in nature. Asexual spores may be d within a closed vessel or spore case known as a "sporangium" *m*, "case"), which is supported by a sporangiophore (*phore*, r") in the case of the genera *Mucor* and *Rhizopus*, members of the Phycomycetes, or they may arise from special hyphae, known nidiophores." In the latter case, the spores are known as "co- "

xual spores are borne in various other ways. The reader interested s aspect of mycology is referred to some of the texts listed at the this chapter.

chlamydospore is a spore with thickened walls and generally is d from a vegetative cell.

ospores are sexual spores and are produced characteristically in a lized sac, or ascus, by the class Ascomycetes.

ospores (*zygo*, "yoke") are spores with thick walls formed as the of the conjugation of two terminal hyphae arising from different es, these colonies representing plus and minus strains of the species. pores are thus sexually produced spores.

tinction is made between molds and a vast number of fungi that e causative agents of plant diseases and thus live an essentially tic life. From the standpoint of industrial microbiology, the term " is generally given to aerobic saprophytes that grow on organic r or solutions with the formation of expansive masses of mycelium, may be thin and superficial in character or which may occur as masses of tough and/or semigelatinous nature. The mycelia can ate the substrate for some distance, especially when growing on r tissues or amorphous masses of material.

number of known species of molds is large, but as their classifica- often difficult because of great differences in appearance on differ- ostrates it is unwise to attempt exact figures.

ds are especially characterized by the ability to elaborate a great of enzymes, and this physiological qualification undoubtedly ts for their ability to thrive on so many materials and in the e of very small amounts of organic matter. Enzyme production tain species will be considered more fully in a later section of this r.

**Growth Requirements of Molds.** Certain elements are essential growth of molds, such as nitrogen, carbon, hydrogen, oxygen, potassium, phosphorus, magnesium, and other elements. Some require special organic substances, such as thiamin, for growth.

*ogen Sources.* Molds, in general, may utilize a large number of n-containing compounds. These differ in relative value, some ting growth by yielding nutritive substances, and some being lly favored on account of their effect in producing large yields of end products. The type of nitrogen compound selected is thus h importance to the fermentologist, who is interested not only in ld but also in the recovery of the end product in as pure a condi- d as free from color as possible.

In general, ammonium salts, nitrates, proteins, peptones, amino and urea are satisfactory sources of nitrogen.

*Carbon Sources.* Molds obtain their energy essentially from ca containing compounds. In the complete breakdown of a carbohy to carbon dioxide and water, as in aerobic respiration, a relatively amount of energy is liberated. In the anaerobic or partial anae breakdown of carbohydrates, intermediate products are formed w possess less energy value than the original carbohydrate. The en thus liberated is less than that evolved during aerobic respiration. type of energy reaction is the one commonly associated with fermenta

Many carbon-containing compounds have been examined as sou of carbon by molds. For example, *Aspergillus oryzae* utilized 51 c pounds, principally alcohols and acids, for growth and respiration (Tan 1932). Olive, linseed, and walnut oils; triolein; pentosans; amylopec cellulose; some higher paraffins (Birkinshaw, 1937); starches; sug and other compounds have been utilized by different molds, in addi to alcohols and acids.

*Other Elements.* Iron, zinc, copper, manganese, molybdenum, gallium appear to be important elements for the growth of certain mo in particular, *Aspergillus niger* (Steinberg, 1934, 1935, 1937). These ments constitute some of the "trace elements."

**Growth Media.** Most molds grow well in a medium containing appropriate starch or sugar, a usable source of nitrogen, and salts su plying essential mineral elements. An acid reaction is desirable.

Media may be classified as synthetic and natural media. Natu media are obviously the tissues or juices of plants or animals in th native state. It is highly desirable, however, especially for identificati purposes, to cultivate molds in media that can be duplicated exactly any time or in different parts of the world. Such synthetic media prepared with pure sugars and chemically pure inorganic or organic co pounds. Steinberg (1939) has reviewed the subject of the growth fungi in synthetic nutrient solutions. This excellent paper contains ma references to the nutrition of fungi.

*Raulin's Medium.* One of the oldest synthetic media is that of Raul which had the following unduly complex composition:

| | G. |
|---|---|
| Water | 1,500 |
| Sucrose | 70.0 |
| Ammonium nitrate | 4.0 |
| Tartaric acid | 4.0 |
| Ammonium phosphate | 0.6 |
| Potassium carbonate | 0.6 |
| Magnesium carbonate | 0.4 |
| Ammonium sulfate | 0.25 |
| Zinc sulfate | 0.07 |
| Ferrous sulfate | 0.07 |
| Potassium silicate | 0.07 |

The foregoing medium has a highly acid reaction (the pH is approx mately 2.9) (Smith, 1938).

*ck's Medium.* For growth and isolation of molds Czapek's medium y used. This has the following composition, as modified by Dox om (Thom and Church, 1926):

| | |
|---|---|
| Sucrose | 30.0 g. |
| Sodium nitrate ($NaNO_3$) | 2.0 g. |
| Potassium phosphate ($K_2HPO_4$) | 1.0 g. |
| Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) | 0.5 g. |
| Potassium chloride (KCl) | 0.5 g. |
| Ferrous sulfate ($FeSO_4 \cdot 7H_2O$) | 0.01 g. or trace |
| Agar | 15 (12–20) g. |
| Water | 1,000 ml. |

l reaction of the preceding medium is neutral or slightly acid. By otassium dihydrogen phosphate ($KH_2PO_4$) instead of potassium drogen phosphate ($K_2HPO_4$) a definitely acid reaction is obtained. preferred by some workers.

se or other sugars, in varying quantities, may be substituted for in the foregoing formula, thus obtaining a medium with wider tions. *Mucors* do not grow well on Czapek's medium containing for they do not readily utilize this sugar.

der to avoid a browning of the medium or the production of y, the phosphates should be dissolved separately in a small portion water, sterilized, and added to the main portion of the sterilized

*Medium.* A medium, liquid or solid, prepared from malt extract l where the cultivation of molds and yeasts is concerned. Such a has been recommended for the determination of molds and yeasts r.

nalt extract agar is prepared by dissolving 30 g. of dehydrated tract and 15 g. of agar in 1,000 ml. of distilled water and auto- at 15 lb. pressure for 20 min. The final reaction is 5.5.

*raud's Medium.* Parasitic molds grow well in a medium that is a ation of the original formula of Sabouraud. Sabouraud's dextrose repared by dissolving 10 g. of peptone, 40 g. of dextrose, and 15 g. in 1,000 ml. of distilled water and autoclaving the medium at essure for 20 min. The final reaction is 5.6.

raud's maltose agar is prepared in the same manner except that is used instead of dextrose. Maltose is more satisfactory for the ion of certain pathogenic molds than dextrose, for example, of *oron audouini* and of *M. lanosum*.

d media are prepared by omitting the agar. Such media are very n certain types of work with molds.

*ral Media.* Prune, potato, carrot, bean, wort, and other juices cts with or without agar are sometimes useful in the cultivation

ent agar or gelatin, such as is used for the growth of bacteria, may hed with carbohydrates and adjusted to an acid reaction.

urther data concerning media, the reader is referred to standard d publications on mycology.

**Methods of Isolating Molds.** There are several methods for iso molds in pure culture. Some of these are similar to methods used i lating bacteria or yeasts.

*By Agar Dilution.* A series of bacterial culture tubes, perhaps to six, each containing about 10 ml. of a suitable agar medium, is he in a water bath to melt the agar. The contents of the tubes are co to 44 to 42°C. and maintained at that temperature in a water bath the tubes are used. At this temperature the agar will not solidify will it cause injury to the mold. A small amount of the mold-contai material is added to the first of the series of tubes. It is mixed thorou with the agar by agitation, and a small loopful of this seeded aga transferred to the second tube of the series. The first tube of agar is poured aseptically into a petri dish. The contents of the second tube shaken carefully, one loopful being transferred to the third tube and rest poured into a petri dish. This process continues until 4 to 6 or m plates have been poured. The culture is diluted by this method, and least one of the plates should contain the desired mold in such dilu that a pure culture may be secured. Agar slopes (slants) may be inc lated from this colony.

An alternate method is similar to the above in that tubes of agar melted and cooled to 44 to 42°C. The culture is added to the first tu and after the tube is shaken carefully, the contents are plated. T second tube of agar is then added to the first tube, which contains a mold spores in the agar still adhering to the sides and bottom of the tu The latter tube is shaken and the contents poured into a petri dish. third, fourth, fifth, and perhaps other tubes of agar are added successiv to the first tube in the same fashion, and the agar is poured.

*By Picking Spores from a Single Spore Head.* In this method, t mycologist selects a colony of the mold that he believes is a pure cultu and, using a hand lens or the low power of the microscope, picks mo spores from a single spore head with a sterile needle and transfers th to a tube containing a medium favorable for growth.

If the plate from which the mold colony is selected contains oth types of molds, there is a possibility of obtaining a mixed culture.

*By the Micromanipulator.* This method, though practicable, requir a certain amount of experience. Excellent results may be obtained the use of this method.

*By the Germination of a Single Spore.* A dilution of spores is made sterile water or saline until a drop contains just one spore (ascertain by an examination of droplets on a slide on the stage of a microscope Droplets are then placed in isolated portions on the surface of agar, the position being marked in order to locate the correct culture in case t plate contains a contaminant.

*By a Modification of the Keitt Single-spore Method.* In Ezekial (1930) modification of the Keitt single-spore method, a nutrient aga selected for growing the desired mold, is melted, poured into a petri dis to form a thin layer, and permitted to solidify. Using a needle (with spatulate tip) infected with the spore-containing material, which ha

luted properly, four to five parallel streaks are made on the surface
agar. The dish is then inverted, incubated for 16 to 24 hr., and
ed through the bottom with the aid of the 16-mm. objective of a
ope in order to locate a sporeling. When a sporeling has been
d, its position is marked with ink on the plate, and it is examined
horoughly with a high magnification of the microscope. Using a
with a cylindric tip, a disk of agar containing the sporeling is
, placed on a thin portion of the agar on an agar slope, and
xamined microscopically to make sure that only one sporeling is
.

*he Hansen Method.* In another method (Hansen, 1926) a dilute
ion of spores is prepared in agar, the agar then being sucked into
y tubes, the diameters of which are not much greater than the
ers of the spores. The capillary tubes are examined under the
ope. When an isolated spore is found, the tube is broken in such
ner that the segment contains a single spore. The glass is treated
cohol and then placed into fresh medium. Growth emerges from
e, and a colony develops. This method operates successfully with
olored spores but not well with small spores.

e-spore isolations may be made also by the Hansen method or by
dner method (refer to Chap 2).

**Identification of a Mold.** The basis for the identification of a
an accurate and complete description of the organism. Informa-
tained by the use of the naked eyes, the hand lens, and the micro-
in the manner outlined in the following paragraphs, is usually
nt.

desirable to cultivate the mold on different types of solid media
to ascertain the medium best adapted to its growth. Ordinarily
's medium is selected to study the characteristics of a mold that
used for industrial purposes. This medium has been used in
g the aspergilli and the penicillia by Thom, Church, Raper, and
in studying the Actinomycetes by Waksman; and in studying other
A large number of molds grow satisfactorily on this medium. For
e of comparative purposes it is a good plan to use this medium,
the mold grows well on it.

idual colonies on Czapek's agar or other medium may be studied
e naked eyes, the hand lens, and the low magnification of the
ope. The following information may be obtained from such obser-
: the rate of growth; the appearance or growth habit; the nature,
ment, size, and color of the fruiting bodies and hyphae; the ele-
and density of different parts of the colony; the presence or absence
thecia; variation in the shape and sizes of the mold heads; and
ata.

plate may be turned over and the color of the underside of the
bserved, also any coloration produced in the medium.

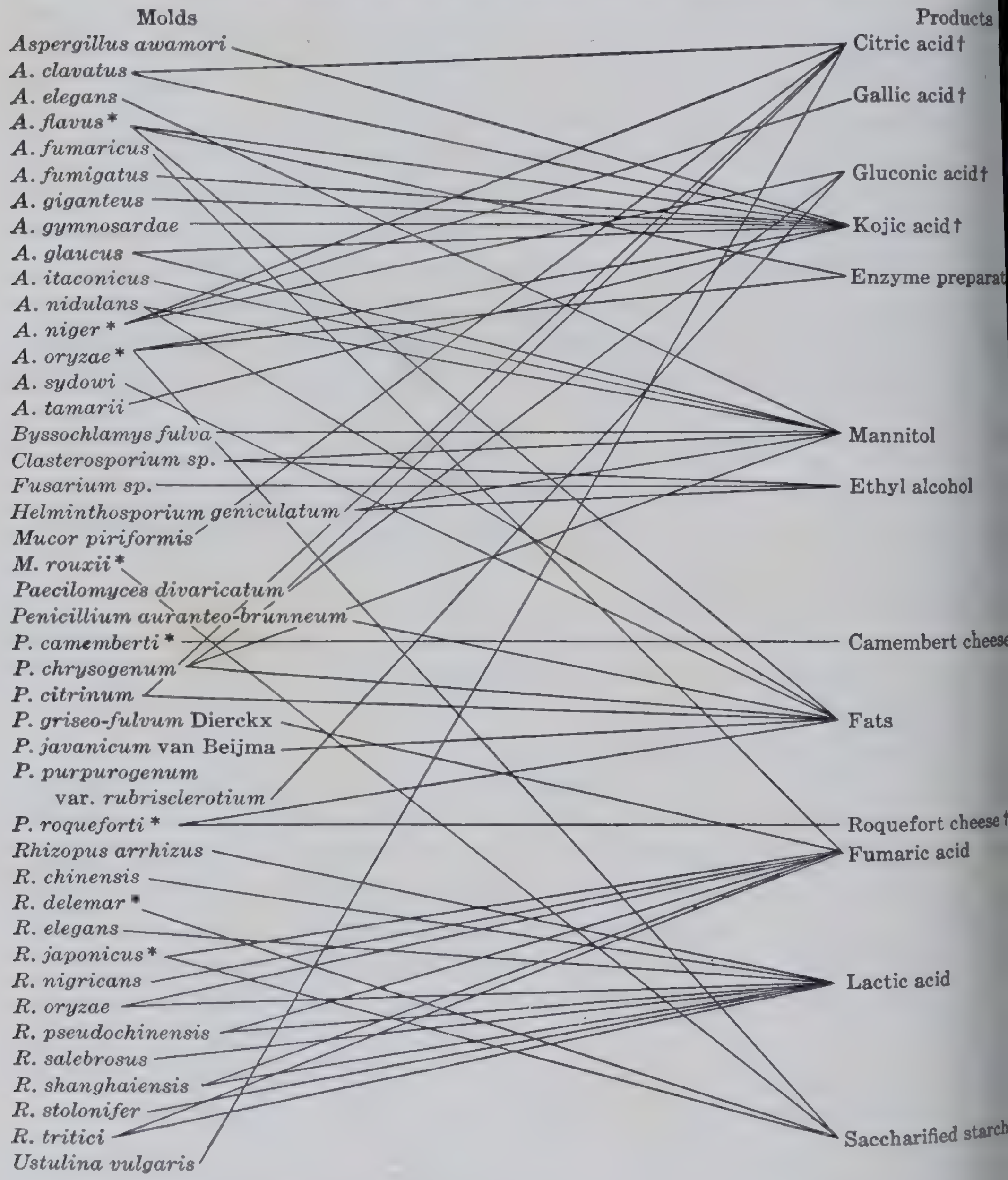
information gained from the foregoing study may be sufficient
ify the mold in so far as the class or order is concerned, but further
ith the aid of the oil-immersion lens of the microscope is usually

necessary in order to obtain enough information to identify th according to genus and species.

Slide cell cultures are very helpful in studies involving the minute ture of molds. Such cultures may be examined either stained or unst The following observations are made on the spores: the shape (averages and extremes), color, markings, and arrangements. F hyphae are examined for branching, septation, width, color, mar and the nature of the walls, whether smooth, pitted, or warted. Cr of chemical compounds or juices elaborated by the mold shou observed.

On the basis of the descriptions thus obtained, an attempt ma made to identify the mold, using a text which describes the genera.

Table 144. Some Molds and Some Mold Metabolic Products *


Molds
Aspergillus awamori
A. clavatus
A. elegans
A. flavus *
A. fumaricus
A. fumigatus
A. giganteus
A. gymnosardae
A. glaucus
A. itaconicus
A. nidulans
A. niger *
A. oryzae *
A. sydowi
A. tamarii
Byssochlamys fulva
Clasterosporium sp.
Fusarium sp.
Helminthosporium geniculatum
Mucor piriformis
M. rouxii *
Paecilomyces divaricatum
Penicillium auranteo-brunneum
P. camemberti *
P. chrysogenum
P. citrinum
P. griseo-fulvum Dierckx
P. javanicum van Beijma
P. purpurogenum var. rubrisclerotium
P. roqueforti *
Rhizopus arrhizus
R. chinensis
R. delemar *
R. elegans
R. japonicus *
R. nigricans
R. oryzae
R. pseudochinensis
R. salebrosus
R. shanghaiensis
R. stolonifer
R. tritici
Ustulina vulgaris
Products
Citric acid †
Gallic acid †
Gluconic acid †
Kojic acid †
Enzyme preparat
Mannitol
Ethyl alcohol
Camembert chees
Fats
Roquefort cheese †
Fumaric acid
Lactic acid
Saccharified starch

* Mold species of present industrial importance.

† Products produced on an industrial basis by mold action.

**ortant Genera of Molds.** From an industrial standpoint, species genera *Aspergillus*, *Penicillium*, *Rhizopus*, and *Mucor* are by far st important at the present time. Table 144 summarizes some of ducts manufactured on a large scale by the action of molds. It noticed that *Aspergillus niger* is particularly important, strains organism being used in three important industrial fermentations—ric, gallic, and gluconic acid fermentations. From among the ia are species which are important in the manufacture of anti- and the ripening of cheeses and which may be used to produce c and citric acids. Species of the genus *Rhizopus* and the genus are of importance in the saccharification of starchy materials.

**ucts Formed by Molds.** Table 145 lists some of the products by molds. Many of these are of a complex nature. Additional ation relating to the metabolic products formed by molds will be n Chap. 42.

**acteristics of the Genus Aspergillus.** The mycelium consists of branching hyphae, which may be brightly colored or colorless ich may produce crusts or sclerotia (Thom and Church, 1926). ycelium is usually par- ubmerged in the substrate tially aerial.

foot cell is a specialized, d, thick-walled cell that se to a fertile hypha, i.e., lk or conidiophore. The l is usually, but not al- submerged in the sub-

stalk, or conidiophore, pproximately perpendic- the long axis of the foot s walls may be smooth, r rough. It may be sep- unseptate. At the apex, lk usually enlarges to vesicle.

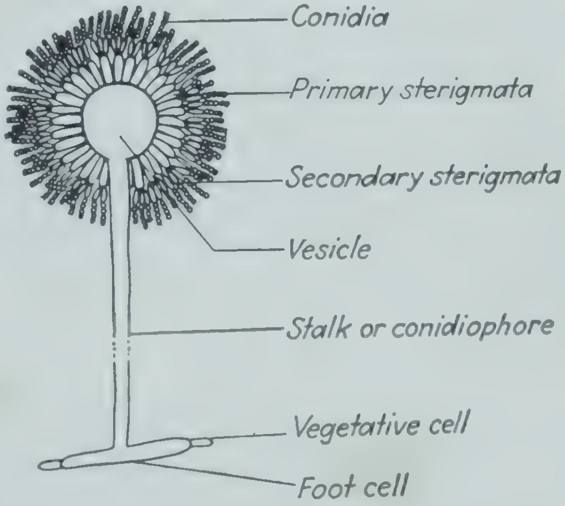


FIG. 88. Diagram of *Aspergillus*. (*Adapted from Thom and Church, "The Aspergilli," The Williams & Wilkins Company, Baltimore*, 1926.)

vesicle, which supports gmata, is globose, hemispherical, elliptical, clavate, calyptrate, or shape. A portion or all of its surface is covered with sterig-

gmata (singular, sterigma) produce conidia or clusters of other ata. When there are two series of sterigmata present, the first he one adjacent to the vesicle, is designated as the "primary ata." These give rise to and support the second series of sterig- hich are called "secondary sterigmata." In the latter case, conidia uced by the secondary sterigmata.

onidium (spore) is produced by an elongation and cell division erigma. A crosswall appears and the newly formed cell matures.

Table 145. Some Products Formed by Molds

| Acids | Alcohols | Enzymes | Pigments | Polysaccharides | Sterols | Miscellaneous |
|---|---|---|---|---|---|---|
| Acetic | Erythritol | Aconitase | Aloe-emodin | Capreolinose | Cholesterol | Acetaldehyde |
| Aconitic | Ethyl | Aldolase | Aspergillin | Galactocarolose | Ergosterol | Alboleersin |
| Agaricic | Gentisyl | Amidase | Atrovenetin | Glycogen | Fungisterol | Antibiotics |
| Allantoic | Glycerol | Aminopeptidase | Aurantin | Gums | Phytosterol | Arsenic compounds |
| Aspergillic | Mannitol | α-Amylase | Aurantiogliocladin | Luteic acid |  | Methyldiethyl arsine |
| Alternaric | Acids (*cont.*) | Carboxypeptidase | Aurofusarin | Mannocarolose |  | Dimethylallylarsine |
| Biformic | Lactic | Catalase | Auroglaucin | Mycodextrin |  | Trimethylarsine |
| Byssochlamic | Linoleic | Cellobiase | Boletol | Polygalactose |  | Chlorine-containing compounds |
| Carlic | Luteic | Cellulase | β-Carotene | Polymannose |  | Caldariomycin |
| Carlosic | Malic | Condensing enzyme | Carviolacin | Rugulose |  | Erdin |
| Carolic | Malonic | Cytase | Carviolin | Sclerotiose |  | Geodin |
| Carolinic | Mannonic | Dextrinase | Catenarin | Starch (mold) |  | Griseofulvin |
| Citric | Marasmic | Dipeptidase | Chrysogenin | Varianose |  | Nalgiolaxin |
| Cyclopaldic | Methylsalicylic | Emulsin | Chrysophanol |  |  | Sclerotiorin |
| Cyclopolic | L-γ-Methyltetronic | Erepsin | Citrinin |  |  | Ustin |
| Dehydrocarolic | Mineoluteic | Fumarase | Citromycetin |  |  | Dimethylselenide |
| Dihydrogladiolic | Mycophenolic | Gentianase | Corticrocin |  |  | Ergot |
| 3,5-Dihydroxyphthalic | Nemotinic | Gentiobiase | Culmorin |  |  | Ergosteryl palmitate |
| Dimethylpyruvic | Norcaperatic | α-Glucosidase | Cynodontin |  |  | Ethyl acetate |
| Emodic | Nudic | β-Glucosidase | Emodic acid |  |  | Hydroxylamine |
| L-Ethylene oxide α,β-dicarboxylic | Odyssic | Glucose-6-phosphate dehydrogenase | Endocrocin |  |  | Lipids |
| Formic | Oxalacetic | Hemicellulases | Erythroglaucin |  |  | Palitantin |
| Frequentic | Oxalic | Hexosephosphate isomerases | Flavoglaucin |  |  | Phenylethylamine |
| Fulvic | Penetrinic | Inulase | *Frangula*-emodin |  |  | Terrein |
| Fumaric | Penicillic | Invertase | Fulvic acid |  |  |  |
| Fusaric |  |  | Fumigatin |  |  |  |
| Fusarinic |  |  | Funiculosin |  |  |  |
|  |  |  | Gliorosein |  |  |  |

Glaucic
Glauconic
Gluconic
Glucuronic
Glycolic
Glyoxylic
Grisic
Helvolic
Homoprotocatechuic
Hydroxyaspergillic
2-Hydroxymethylfurane-5-carboxylic
Isovaleric
Itaconic
Itatartaric
Kojic
γ-Ketopentadecoic

Stipitatic
Succinic
Terreic
Terrestic
Thelophoric
Trihydroxyphenylglyoxylic
Ungulinic
Ustic
Ustilagic

Lecithinase
Lipase
Malic dehydrogenase
Malic enzyme
Maltase
Melezitase
Notatin
Nuclease
Pectinesterase
Penicillinase
Phosphoglucomutase
Polygalacturonase
Proteases
Protopectinase
Pyruvic oxidase
Raffinase
Rennet
Succinoxidase
Sulfatase
Tannase
Transacetylase
Transketolase
Trehalase
Triosephosphate isomerase
Urease

Luteoleersin
4-Methyltoluquinone
Monascoflavin
Monascorubin
Muscarufin
Nalgiolaxin
Nalgiovensin
Ochracin
Oösporin
Penitrinic acid
Penicillopsin
Phoenicin
Physcion
Ravenellin
Rhein
Roseopurpurin
Rubrofusarin
Rubroglaucin
Spinulosin
Sulochrin
Thelophoric acid
Tritisporin

Other conidia are produced by the same sterigma in a similar r with the result that an unbranched chain of conidia appears, of the outermost are the eldest. These conidia vary among different in respect to color, size, and shape.

Spore heads vary in respect to arrangement, color, size, and Heads may be globose, like *A. niger;* hemispherical, like *A. terrico americana*; elliptical; clavate, like *A. clavatus* (the head in this clavate or elliptical); columnar, like *A. flavipes;* or of some other c teristic shape.

Perithecia are produced by only a few species. The peritheciun thin-walled receptacle, or fruit body, commonly globose or flask-sh closed at maturity, which produces ascospores. The ascospores ar erated when the thin walls of the perithecium break.

Sclerotia, which are hard masses formed from the mycelium, us possess somewhat characteristic markings and colorations. They are duced by some species of *Aspergillus.*

For a further discussion of this subject the reader is referred t Manual of the Aspergilli," by Thom and Raper (1945).

**Some Important Aspergilli.** Members of the *A. flavus-oryzae* g especially strains of the *A. oryzae* series, have large industrial importa particularly in the Orient. In Japan, *A. oryzae* is used to saccha rice starch in the manufacture of sake and other alcoholic liquors; in manufacture of *shoyu* (soy sauce); in the manufacture of *miso*, a soy product used as a breakfast food; and in the preparation of *mizaum* sugar sirup made from rice. *A. oryzae* is used also in the preparatio enzyme mixtures, which appear on the market under such trade name Takadiastase, Polyzyme, Digestin, Oryzyme, and Kashiwagidiast Kojic acid is produced by the same mold.

Strains of *A. niger* are used in two industrial fermentations (r to Chaps. 28 and 29). This mold may cause serious damage in the te industry.

*A. tamarii* is used in the Orient in the production of *tamari* sauce f soybeans or from soybeans mixed with rice.

**Divisions of the Penicillia.** The penicillia are divided into four m groups by Thom (1930): the *Monoverticillata*, the *Biverticillata-s metrica*, the *Polyverticillata-symmetrica*, and the *Asymmetrica*. Th groups are still further divided in some cases. The basis for the princ divisions is the type of branching in the penicillus or spore head. Col characteristics furnish a basis for further subdivision.

Figure 89 shows a division of the penicillia proper into main gro Some important species are listed.

The *Monoverticillata* contain one cluster, whorl, or verticil of ste mata (the conidia-producing organs of the mold) supported by conidiophore. In this group belong the *Citromyces* of Wehmer, mo that have the ability to produce citric acid; and *P. Charlesii* and *spinulosum*, molds studied for their biochemical characteristics by R trick and his associates.

In the *Biverticillata-symmetrica*, a verticil or whorl of sterigmat

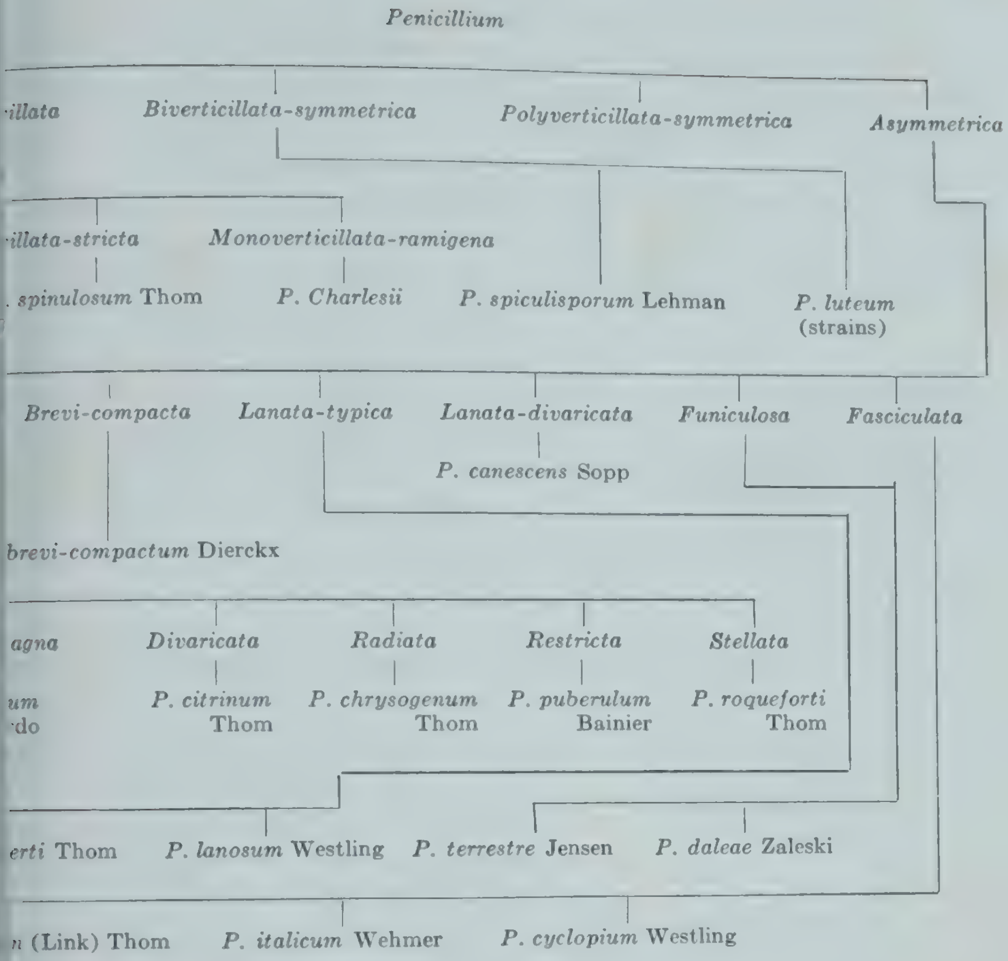

Penicillium
·illata
Biverticillata-symmetrica
Polyverticillata-symmetrica
Asymmetrica
·illata-stricta
Monoverticillata-ramigena
. spinulosum Thom
P. Charlesii
P. spiculisporum Lehman
P. luteum (strains)
Brevi-compacta
Lanata-typica
Lanata-divaricata
Funiculosa
Fasciculata
P. canescens Sopp
brevi-compactum Dierckx
agna
Divaricata
Radiata
Restricta
Stellata
um
·do
P. citrinum Thom
P. chrysogenum Thom
P. puberulum Bainier
P. roqueforti Thom
erti Thom
P. lanosum Westling
P. terrestre Jensen
P. daleae Zaleski
n (Link) Thom
P. italicum Wehmer
P. cyclopium Westling

FIG. 89. A subdivision of the genus *Penicillium*.

d by short branches or metulae (singular, metula), which are
l symmetrically about the axis of the conidiophore in the form of
l or whorl. *P. luteum-purpurogenum*, a mold that produces glu-
id, and *P. pinophilum*, a mold that stains wood, are members of
p.

are three or more stages of branching in the *Polyverticillata-*
*ica* group, which contains only a few unimportant molds.

*Asymmetrica*, the spore head, or penicillus, is branched asym-
ly about the axis of the conidiophore. This group is the largest
ur and includes species of economic importance: molds producing
ristic changes in cheeses; molds causing destruction of fruits; and
ith the ability to produce gluconic acid or other compounds from
glucose solutions.

pecies of *Penicillium*, *P. italicum* and *P. digitatum*, cause much
to citrus fruits. Invasion of the fruit occurs through wounds.
handling and packing, the use of special protective treatments to
infection of the fruit, and the use of wrapping material treated
nenyl do much to prevent losses caused by these molds.

*icum* produces a soft rot of citrus fruits. The colonies on the
blue-green in color owing to the spores.

*P. digitatum* (*P. olivaceum*) causes the infected fruit to shrivel ar up. Conidia of the mold are dull yellow-green or olive-green in

Soft rot of stored apples and pears is caused by *P. expansum.* Co formation is characteristic of the mold. The coremia, i.e., bundl conidiophores, are green to gray-green in appearance.

**The Genus Rhizopus.** Species of *Rhizopus* are of industrial va man. *R. oryzae* and other species have the ability to produce L(+)- acid from nutrient sugar media (see Chap. 34), while *R. japonicu delemar,* and other species have been in the Amylo process for conve starches to sugars (see page 864).

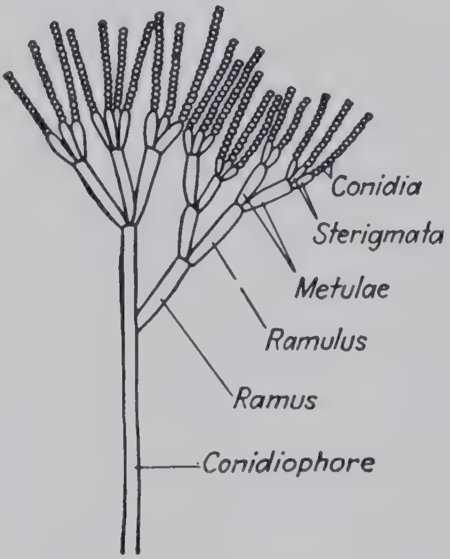


FIG. 90. Diagram of the penicillus of a *Penicillium.* (*Adapted from Thom, "The Penicillia," The Williams & Wilkins Company, Baltimore,* 1930.)

On the other hand, certain speci *Rhizopus* are the cause of the destru of plant products. *R. nigricans* is cause of soft rot in sweet potatoes, e cially during storage. The same mold causes rot in fruits, for example, the watery rot of strawberries, which known also as "leak." *R. necans* prod a bulb rot in the lily; *R. nodosus,* a rot of cotton (Heald, 1943).

*General Characteristics of the Ge* Reference to the accompanying fig will aid in understanding the general scription of the genus *Rhizopus.* E aerial sporangiophores arise from nodes, the places where the stolons or r ners are attached to the substrate or s roundings by means of rhizoids. Sp heads are borne at the upper ends of the fruiting hyphae (sporang phores) in clusters or whorls. Groups of sporangiophores are connec by hyphae of a vegetative nature, the stolons, which may be regarded distributing hyphae. The rhizoids are vegetative hyphae, which fu tion as anchors.

Thick-walled spores (chlamydospores) are formed by some species, example, *R. oryzae.*

**Mold Enzymes.** Various kinds of enzymes are produced by differ species of molds. A list of some of the enzymes produced by strains the *A. niger* and *A. oryzae* groups and *P. camemberti* is given in Ta 146. Other enzymes formed by *A. niger* are referred to in the chapter citric acid production.

With certain exceptions, molds have not been thoroughly studied respect to the kinds of enzymes elaborated and the conditions under wh they are produced. This field of research offers many possibilities.

**Apparatus for Cultivating Molds.** From time to time special equ ment has been devised for cultivating molds or for carrying out m fermentations on a large scale in the laboratory.

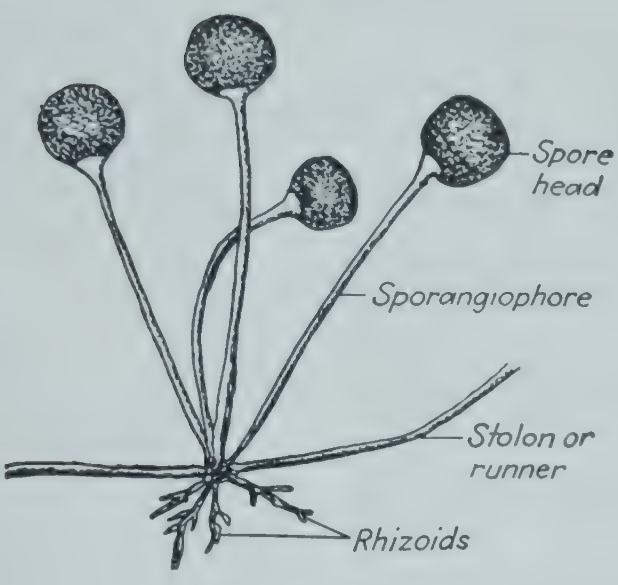


Fig. 91. Diagram of *Rhizopus nigricans.*

146. Some Enzymes Formed by Some Industrially Important Molds

| *Aspergillus niger* | *A. oryzae* | *Penicillium camemberti* |
|---|---|---|
| Amylase (diastase) | Amidase | Amidase |
| Cellobiase | Amylase | Amylase |
| Emulsin | Catalase | Emulsin |
| Gentianase | Cytase | Erepsin |
| Gentiobiase | Dextrinase | Inulase |
| Inulase | Emulsin | Invertase |
| Invertase | $\alpha$-Glucosidase | Lactase |
| Lipase | $\beta$-Glucosidase | Lipase |
| Maltase | Histozyme | Maltase |
| Melezitase | Inulase | Nuclease |
| Nuclease | Invertase | Protease |
| Protease | Lactase | Raffinase |
| Raffinase | Lecithinase | |
| Rennet | Lipase | |
| Tannase | Maltase | |
| Trehalase | Protease | |
| Zymase | Rennet | |
| | Sulfatase | |
| | Tannase | |

and his collaborators (1935) designed a sterilizer-incubator (see age 530).

## References on Molds

tudies on the Classification of the Penicillia, *Jour. Gen. Microbiol.* Japan, d 2): 1–193; 2(3): 195–344 (1956).
os, C. J.: "Introductory Mycology," John Wiley & Sons, Inc., New York,

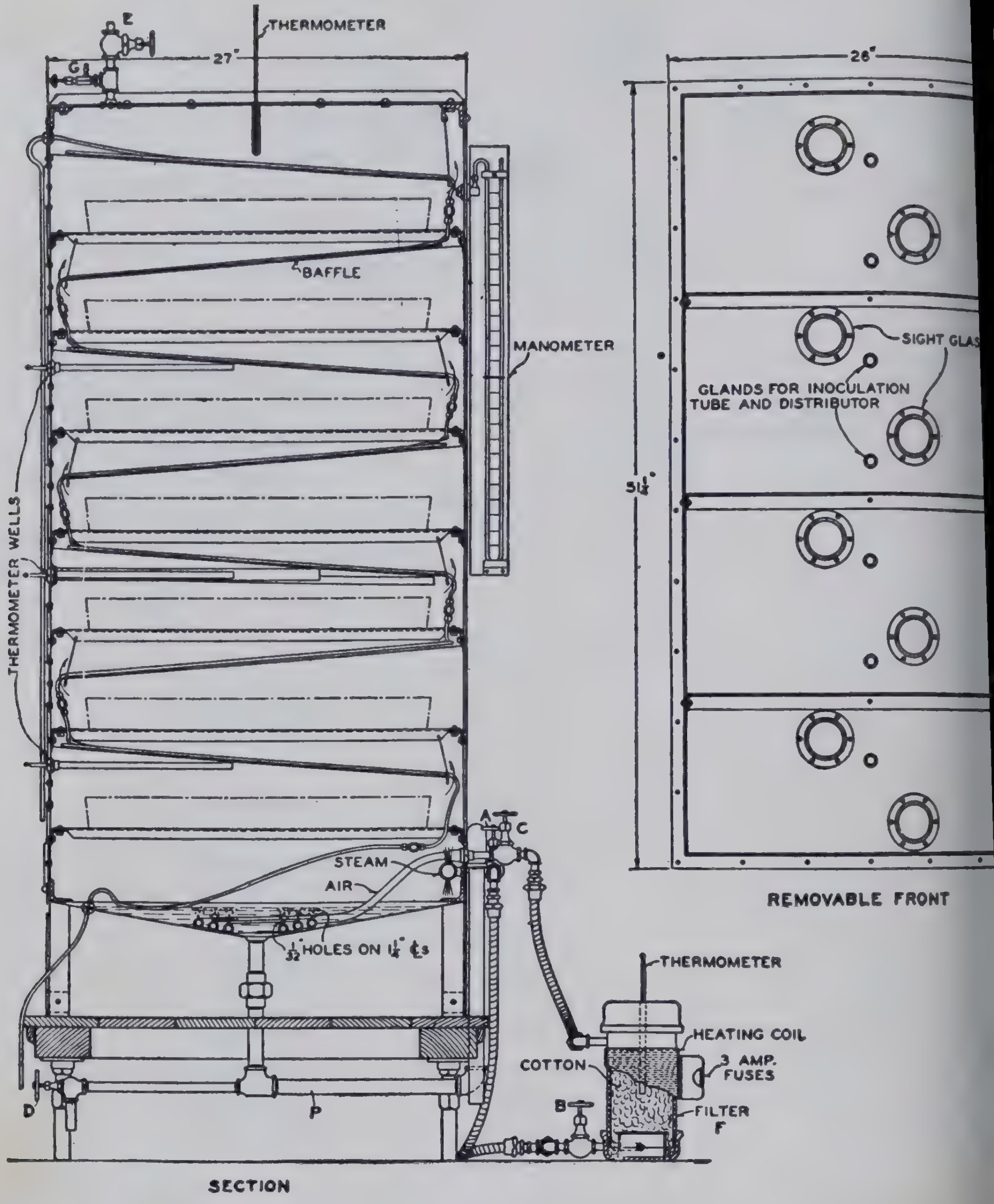

THERMOMETER
E
G
27"
BAFFLE
MANOMETER
THERMOMETER WELLS
STEAM
AIR
$\frac{1}{32}$" HOLES ON 1¼" ℄s
A
C
THERMOMETER
HEATING COIL
COTTON
3 AMP. FUSES
B
FILTER F
D
P
SECTION
26"
SIGHT GLAS
GLANDS FOR INOCULATION TUBE AND DISTRIBUTOR
51¼"
REMOVABLE FRONT

Fig. 92. Cabinet incubator. [*Courtesy of G. E. Ward, L. B. Lockwood, O. E.* *and H. T. Herrick, Ind. Eng. Chem.,* 27: 318 (1935). *Copyright* 1935 *by the Amer* *Chemical Society and reprinted by permission of the copyright owner.*]

Barnett, H. L.: "Illustrated Genera of Imperfect Fungi," Burgess Publishing C pany, Minneapolis, 1955.

Bessey, E. A.: "A Textbook of Mycology," McGraw-Hill Book Company, Blakiston Division, New York, 1935. (This book contains a chapter enti "Guide to the Literature for the Identification of Fungi," which should prov much value. An extensive bibliography is presented.)

Biourge, Ph.: Les moisissures du groupe Penicillium Link, *Cellule,* 33 (fasc. 7–330 (1923).

Birkinshaw, J. H.: *Biol. Rec., Cambridge Phil. Soc.,* 12: 357 (1937).

Clements, F. E., and C. L. Shear: "The Genera of Fungi," The H. W. Wilson C pany, New York, 1931.

De Bary, A.: "Comparative Morphology and Biology of the Fungi, Mycetozoa Bacteria," Oxford University Press, New York, 1887.

C. W.: "Medical Mycology," The C. V. Mosby Company, St. Louis, 1935.
J. A.: Taxonomic characters of the genera *Alternaria* and *Macrosporium*,
*Jour. Botany*, **4**: 439 (1917).
W. N.: *Phytopathology*, **20**: 583 (1930).
ick, H. M.: "The Lower Fungi—Phycomycetes," McGraw-Hill Book Com-
, Inc., New York, 1930.
S.: "Practical Mycology Manual for Identification of Fungi," Brøggers Boktr.
ag, Oslo, Norway, 1953.
n, E. A., and C. W. Dodge: "Comparative Morphology of Fungi," McGraw-
Book Company, Inc., New York, 1928.
J. C.: "A Manual of Soil Fungi," Iowa State College Press, Ames, Iowa,
.
-Vaughan, H. C. I.: "Fungi," The Macmillan Company, New York, 1922.
nd B. Barnes: "The Structure and Development of Fungi," 2d ed., The Mac-
n Company, New York, 1938.
H. N.: *Science*, **64**: 384 (1926).
rger, J. W.: "Mycology and Plant Pathology," McGraw-Hill Book Company,
Blakiston Division, New York, 1917.
L. E.: "The Physiology of Reproduction in Fungi," Cambridge University
, London, 1957.
. D.: "Introduction to Plant Pathology," 2d ed., McGraw-Hill Book Com-
, Inc., New York, 1943.
L. C. C.: "The Mushroom Handbook," The Macmillan Company, New
, 1936.
.: "Handbuch der technischen Mykologie," 2d ed., Gustav Fischer Verlags-
handlung, Jena, Germany, 1904–1914.
'Technical Mycology," Charles Griffin & Co., Ltd., London, 1898–1910.
G., and H. L. Barnett: "Physiology of the Fungi," McGraw-Hill Book
pany, Inc., New York, 1951.
.: "Kryptogamenflora für Anfänger," Band II, "Die mikroskopischen Pilze,"
., Springer-Verlag, Berlin, Vienna, 1922.
W. H., L. M. Pruess, H. J. Gorcia, and H. C. Greene, *Ind. Eng. Chem.*,
13 (1933).
st, L.: "Kryptogamenflora von Deutschland, Oesterreich und der Schweiz,"
rd Kummer, Leipzig, 1884–1921.
om, J.: "Fungi," Ernest Benn, Ltd., London, 1929.
he Uses of Fungi, address to Section K (Botany) of the British Associa-
for the Advancement of Science at Blackpool, Sept. 10, 1936, *Ann. Rept.*,
p. 189.
. B., and D. F. Alexander: Preservation of Molds by the Lyophil Process,
*logia*, **37**(4): 499–525 (1945).
d C. Thom: "A Manual of the Penicillia," The Williams & Wilkins Com-
Baltimore, 1949.
P. A.: "Fungi Italici autographice delineati," Pavia, 1877–1886.
Sylloge fungorum omnium hucusque cognitorum," Pavia, 1882–1931.
J.: "Fundamental Principles of Bacteriology," 4th ed., McGraw-Hill Book
pany, Inc., New York, 1954.
W.: Fungi and Fungous Diseases, *Stuart Circle Hosp. Bull.*, August, 1932.
C. E., C. W. Emmons, and H. M. Tsuchiya: "Henrici's Molds, Yeasts, and
omycetes," 2d ed., John Wiley & Sons, Inc., New York, 1947.
: "An Introduction to Industrial Mycology," Edward Arnold & Co., London,

H., and E. A. Dick: "A Glossary of Mycology," Harvard University Press,
ridge, Mass., 1957.
R. A.: The So-called "Chemical Stimulation" of *Aspergillus niger* by Iron,
nd Other Heavy Metal Poisons, *Bull. Torrey Botan. Club*, **61**: 241 (1934).
he Nutritional Requirements of the Fungus *Aspergillus niger*, *Bull. Torrey*
. *Club*, **62**: 81 (1935).

———: Nutrient-solution Purification for Removal of Heavy Metals in D
Investigations with *Aspergillus niger, Jour. Agr. Research,* **51**: 413 (1935

———: Role of Molybdenum in the Utilization of Ammonia and Nitrate Nitr
*Aspergillus niger, Jour. Agr. Research,* **55**: 891 (1937).

———: Growth of Fungi in Synthetic Nutrient Solutions, *Botan. Rev.,* **5**: 327

Stevens, F. L.: "Plant Disease Fungi," The Macmillan Company, New York,

Tamiya, H.: *Acta Phytochim. Japan,* **6**: 1 (1932).

Thom, C.: "The Penicillia," The Williams & Wilkins Company, Baltimore,

——— and M. B. Church: "The Aspergilli," The Williams & Wilkins Co
Baltimore, 1926.

——— and M. Phillips: Chemistry—Lignin-like complexes in fungi, *Jour.*
*Acad. Sci.,* **22**: 237 (1932).

——— and K. B. Raper: "A Manual of the Aspergilli," The Williams &
Company, Baltimore, 1945.

Ward, G. E., L. B. Lockwood, O. E. May, H. T. Herrick: *Ind. Eng. Chem.,*
(1935).

Wolf, F. A., and F. T. Wolf: "The Fungi," John Wiley & Sons, Inc., New York
Vols. I and II.

Wollenweber, H. W., and O. A. Reinking: "Die Fusarien," Paul Parey, Berlin

———, C. D. Sherbakoff, O. A. Reinking, H. Johann, and A. A. Bailey:
mentals for taxonomic studies of Fusarium, *Jour. Agr. Research,* **30**: 833 (1

Zycha, H.: "Kryptogamenflora der Mark Brandenberg," Band VIa, "Pilze
"Mucorineae," Verlagsbuchhandlung Gebrüder Borntraeger, Berlin, 1935.

## CHAPTER 28

# THE CITRIC ACID FERMENTATION

roduction of citric acid by fermentation on a commercial basis
a highly important achievement in the field of industrial micro-
It has made the United States self-sufficient in respect to the
id supply and greatly changed the commerce of the world in citric
l calcium citrate.

acid (COOH·CH$_2$·C(OH)·COOH·CH$_2$·COOH) was first iso-
m lemon juice and crystallized as a solid by Scheele in 1784. It is
a natural constituent of citrus fruits, pineapples, pears, peaches,
l other fruits and tissues. The citric acid extracted from these
is known as "natural citric acid" in contrast to "fermentation
id." Cull lemons, limes, and pineapples are the principal sources
al citric acid, which is produced chiefly in Italy, especially Sicily,
in California, Hawaii, and the West Indies.

22, Italy produced approximately 90 per cent of the world's
f calcium citrate, which is used in citric acid manufacture. Much
alcium citrate was exported to the United States, England, and
Since 1927, however, very little calcium citrate or citric acid
imported into this country. Several factors have been responsi-
is change: the production of citric acid by mold fermentation, an
in the numbers of trees bearing lemons in the United States,
ions of concentrated lemon juice, and high import duties.

er, in 1893, first described citric acid as a product of mold fer-
n. Two molds, which he designated as *Citromyces pfefferianus*
*laber* (classified by Thom as penicillia), produced the acid from
sucrose solutions containing calcium carbonate. Later Wehmer
the formation of citric acid by *Penicillium luteum* and *Mucor*
s, but it is interesting to note that he believed that the black
produced only oxalic acid. This idea was disproved by Thom
rie. Nevertheless, oxalic acid is generally an accompaniment of
acid produced.

7, Currie, of the U.S. Department of Agriculture, published the
f a fundamental research concerning the production of citric acid
in of *Aspergillus niger*. Doelger and Prescott, in 1934, corrobo-
results of Currie and made other valuable contributions con-
he fermentation.

erature of the past two decades contains many references to the
l fermentation, but no attempt will be made to review it in detail.
elected references giving the principal facts gained by research

will be found at the end of this chapter. The interested student is to consult some of the papers cited, especially that of Von Lo (1945).

## PRODUCTION OF CITRIC ACID BY SURFACE-CULTURE METH

**Significant Factors in Fermentation.** The organism, the correct relation of the various constituents of the medium—sugar and inor salts—the pH, the ratio of surface area to volume of solution ferme the oxygen supply, and the temperature have much to do with the n and magnitude of the yield of end products recovered from a ferment medium. By adjusting the salts and pH carefully, it is possible to pro citric acid with a negligible or small amount of oxalic acid.

*Organisms.* Since the historic researches of Wehmer, it has been sh that a large number of fungi have the ability to produce citric acid. of the fungi produce small yields; some produce undesirable substan some, on account of their unstable cultural characteristics, woul unsatisfactory for use on a commercial basis. Thus the choice of a st is of great importance.

*Aspergillus niger, A. clavatus, Penicillium luteum, P. citrinum, Pae myces divaricatum, Mucor piriformis, Ustulina vulgaris,* and ano species of *Mucor* have been used to produce citric acid in the labora or on a commercial scale; however, only strains of *A. niger* appear t of industrial importance.

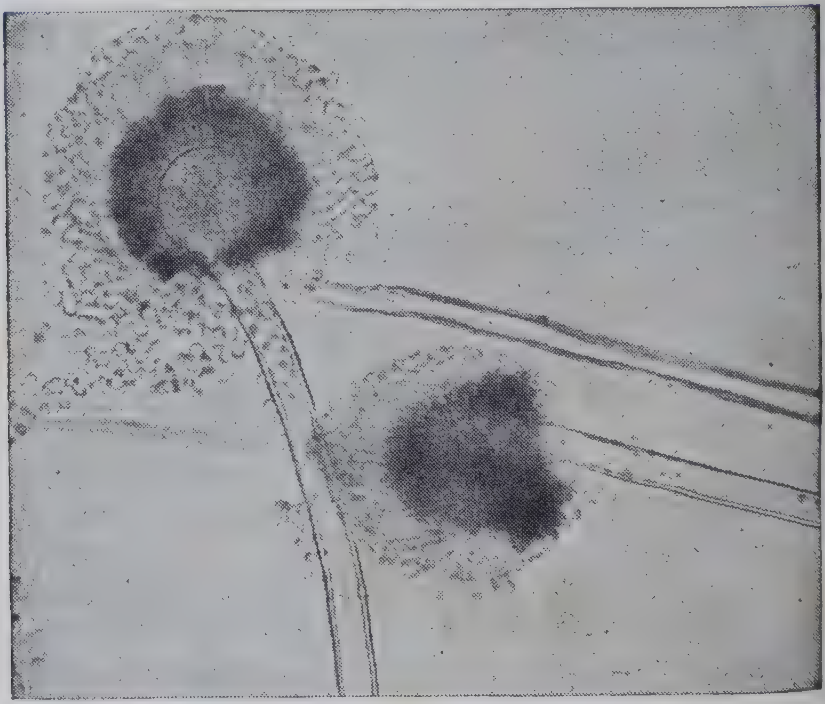


FIG. 93. Typical spore-bearing heads of *Aspergillus niger*. Club-shaped head two rows of columnar cells with spores forming at the ends of the second row of [*From W. P. Doelger and S. C. Prescott, Ind. Eng. Chem.,* 26: 1142 (1934). *Copy 1934 by the American Chemical Society and reprinted by permission of the copy owner.*]

ns of the *A. niger* group of molds have usually given most suc-
results, both in the laboratory and on an industrial basis. Many
molds produce high yields, possess fairly uniform biochemical
eristics, are easily cultivated, and produce a negligible quantity of
able end products.

. Many organic substances, among them 2-, 3-, 4-, 5-, 6-, 7-, and
on compounds (principally sugars), may be fermented to citric
Maximum yields have been secured, usually, from sucrose and
. Occasionally glucose, under certain circumstances, has given
lds, comparable with those from sucrose. For industrial fermen-
sucrose and technical glucose are best; maltose and molasses are
irable.

neral, a high concentration of sugar is required to produce high
f citric acid. Solutions with concentrations of 14 to 20 per cent
used. Currie advocated the use of 125 to 150 g. of sucrose per
Doelger and Prescott obtained highest yields when using a con-
on of 140 g. of sucrose per liter in fermentations that were allowed
or 9 to 12 days. They found that, if more than 15 per cent of sugar
d, a greater amount of sugar than normal (less than 3 per cent)
d unconverted to citric acid. Substitution of part of the sucrose
ose or glucose, such that the concentrations of these sugars repre-
to 5 per cent (out of the total of 14 per cent) resulted in lower
f citric acid than were obtained from controls containing sucrose
. Partial hydrolysis of sucrose during sterilization likewise resulted
yields.

*anic Salts.* In addition to the carbon, hydrogen, and oxygen
by the carbohydrate, also nitrogen, potassium, phosphorus, sul-
magnesium are indispensable in the fermentation medium,
g to Currie, and Doelger and Prescott. Currie (1917) found that
t favorable medium for producing citric acid contained the
g:

| | G./Liter |
|---|---|
| Sucrose | 125 –150 |
| $NH_4NO_3$ | 2 – 2.5 |
| $KH_2PO_4$ | 0.75– 1.0 |
| $MgSO_4 \cdot 7H_2O$ | 0.20– 0.25 |
| HCl to pH 3.4–3.5 (5–4 ml. $N/5$ HCl) | |

r and Prescott (1934) found the following medium to be most
ory, for the strain of *A. niger* used produced high yields of citric
n less than 2 per cent of oxalic acid from this medium:

| | G./Liter |
|---|---|
| Sucrose | 140 |
| $NH_4NO_3$ | 2.23 |
| $K_2HPO_4$ | 1.00 |
| $MgSO_4 \cdot 7H_2O$ | 0.23 |

and sugars are dissolved and made up to 1 liter with distilled
justed to pH 2.20 to 1.60 with $N/1$ HCl, and sterilized at 8 to
am pressure per square inch for 30 min.)

If more than 2.50 g. of ammonium nitrate, 1.50 g. of potassi hydrogen phosphate, and 0.30 g. of magnesium sulfate were us acid formation increased and the yield of citric acid was Ammonium nitrate in a concentration of more than 2.50 g. per lit the formation of a heavy mat. More than 0.30 g. of magnesiu per liter favored sporulation. In general, high yields of citric obtained when the mats were thin and the sporulation light absent; these results were secured when a minimum quantity of salts was used. Restriction of the nitrogen supply tended increased yields of citric acid.

Wells and Herrick (1938) report the following limits for the an salts generally used in the fermentation: 0.03 to 0.1 per cent of 0.01 to 0.05 per cent of $MgSO_4 \cdot 7H_2O$, and 0.16 to 0.32 per $NH_4NO_3$.

In certain cases, other nitrogen-containing salts have been be superior to ammonium nitrate. Sodium nitrate in a 0.4 per c centration was found to be better than ammonium nitrate or am sulfate by Porges, while Butkewitsch and Gaewskaya used po nitrate in a 0.35 per cent concentration in order to obtain high citric acid.

The use of salts other than those mentioned above does not appear to be necessary, although many apparently conflicting sta concerning the value of the use of certain salts in the medium for ing high yields of citric acid appear in the literature. For exampl investigators state that iron and zinc accelerate the formation o acid; others are of the opinion that salts of iron and zinc stimulate of the mycelium without increasing the yields. Still others have that zinc salts have a definite inhibitory effect on citric acid prod Thus, in a few instances, it seems that iron, and possibly zinc salt favored acid production.

Undoubtedly, the strain of fungus used by a worker has a very tant bearing on the salt requirement. That this is true has been by several men. Osnizkaya, using one strain of *A. niger*, obta marked increase in the yield of citric acid from sucrose by the a of 0.3 per cent magnesium nitrate, but, when using a second stra addition of magnesium nitrate actually caused a slight diminution yield. According to Quilico and DiCapua, the effect of iron on acid production depends on the strain of *A. niger* used. In one ca yield of citric acid was increased and in another case decreased addition of increasing quantities of iron.

Perlman, Dorrell, and Johnson (1946) studied the effects of the tion of metallic ions to two highly purified media on the product citric acid by the surface-culture method, using five strains of *A.* They concluded, on the basis of many experiments, that the op concentration of a given metallic ion for the stimulation of citri production varied with the strain of *A. niger* used. For example, the tion of 0.1 mg. of iron per liter to one fermentation medium pr optimum results in the case of *A. niger* 62; the addition of between 0 of iron per liter to a second fermentation medium was optimum ins 62, 69, 70, and 72; and the addition of 10 mg. of iron per liter d to be optimum for strain 59. When other stimulatory ions were gether with iron, the results were in general no better than those d with iron alone. However, in the case of strain 62, some combi- of iron and manganese ions produced better yields than either ne.

inum, chromium, iron, and manganese ions were the only ones mulated acid production with *A. niger* 62. The other ions used, enum, copper, zinc, and calcium, were inhibitory to acid produc- the concentrations used. Molybdenum stimulated acid production n 72, while iron was somewhat inhibitory.

presence or absence of minute traces of elements in a medium may marked effect on the result obtained. Improved methods of analy- use of the spectroscope and other precision measurements—have ne microbiologist and chemist greatly in recent years in detecting sence of unsuspected elements in supposedly pure compounds. An ng amount of evidence stresses the importance of the presence of aces of substances, in quantities of a fraction of a part per million, nple.

The maintenance of a favorable pH is most important for the ul progress and termination of a fermentation. Currie demon- that, by controlling the pH and the inorganic salts, the propor- citric and oxalic acids could be varied considerably. In fact, when nditions that favored the highest yields of citric acid, the forma- oxalic acid was nearly completely suppressed.

se of a low pH is advantageous in that high yields of citric acid red, oxalic acid formation is suppressed, and the danger of con- ion is minimized. Sterilization is more readily effected at a low the laboratory, successful fermentations have been carried out resorting to heat sterilization of the medium when the initial pH (2.20 or below). In general, the best citric-acid-producing molds he greatest tolerance to low pH values.

chloric acid was used by Currie to adjust the pH of his medium 3.5. Doelger and Prescott advocated the use of this acid also, lement chlorine was found to be of distinct value as a constituent edium. The pH range of 1.60 to 2.20 was found to be the most ory by these men; a pH value in the upper part of this range d when distilled water was employed in the medium rather than dge tap water. This indicates that for commercial production racter of the water supply should be carefully observed. From t of view of yields of citric acid, sulfuric, nitric, and acetic acids und to be inferior to hydrochloric acid. A quantity of formic ficient to lower the pH to 3.0 prevented the mold spores from ting.

not generally considered necessary to add calcium carbonate to ze the acids formed during fermentation. Its use favors con- ion, while its absence favors higher yields and a shorter fermentation period. However, calcium carbonate has been used by W Chrzaszcz, and Peyros to increase the yield of citric acid. Here the strain of *A. niger* used and its tolerance to acid may be o significance.

*Ratio of Surface Area to Volume.* In the citric acid fermentati conversion of sugar to citric acid is brought about by intrac enzymes and therefore takes place within the living cells that ma the mycelial mat. Sugar passes by osmosis into the cells, while th diffuses out through the cells. The rate at which enzymic and di processes proceed will determine the length of the fermentation p Obviously, in a deep vessel containing a large volume, the progr acid formation will be relatively slow, for the surface area of th will be small in comparison with the volume. By using shallow p large surface area of mycelium is exposed to a relatively shallow la medium. Conversion of sugar to citric acid proceeds much more ra The ratio of surface area to volume at which a maximum quanti citric acid will be produced during the shortest fermentation period a minimum of sugar unconverted to citric acid should be used.

The effect of varying the ratio of the volume to the surface ar the medium is illustrated in Table 147, from Doelger and Pres

TABLE 147. THE EFFECT OF VARYING THE RATIO OF VOLUME TO SURFACE AREA THE YIELDS OF CITRIC ACID *

| Volume/ surface area ratio, ml./sq. cm. | Original volume, ml. | Sucrose per pan, g. | Final volume, ml. | Citric acid/ 100 ml. g. | Total citric acid per pan, g. | Yie citri to s per |
|---|---|---|---|---|---|---|
| 2.45 | 2,000 | 280 | 1,810 | 6.35 | 114.9 | 41 |
| 2.20 | 1,800 | 252 | 1,620 | 7.05 | 114.2 | 43 |
| 2.08 | 1,700 | 238 | 1,490 | 7.40 | 110.3 | 46 |
| 1.83 | 1,500 | 210 | 1,310 | 7.75 | 110.2 | 48 |
| 1.22 | 1,000 | 140 | 780 | 8.80 | 68.6 | 49 |

* W. P. Doelger and S. C. Prescott, *Ind. Eng. Chem.*, **26**: 1142 (1934). Copy 1934 by the American Chemical Society and reprinted by permission of the copy owner.

researches. Shallow pans of aluminum (25 by 33 cm.), of a purit 99.80 per cent or greater, were used during the experiment. Stan medium at a pH of 2.40 was inoculated and maintained at a tempera of 26°C. until the ninth day, when analyses were carried out.

Agitation of the medium by a gentle or moderate shaking mo retards the rate of citric acid production, according to Doelger and P cott. (Compare with the sorbose and dihydroxyacetone fermentat when the shallow-pan method is used.)

*Oxygen Supply.* Large amounts of air have an adverse effect on yield of citric acid, according to Porges, Doelger and Prescott, and Gu The flow of small amounts of air over the mat has no injurious ef

r. as Wells and his associates have passed sterile, humidified, dioxide-free air over mats in 2,000-ml. Erlenmeyer flasks at a rate l. per min. and obtained results similar to those secured with conceiving no special air supply. Too low an air supply, on the other lso reacts unfavorably on the yield of citric acid. Regulation of of air must therefore be determined experimentally for each tion of apparatus.

*erature.* The temperature used will depend in part on the organ- l the fermentation conditions. Temperatures of 25 to 35°C. are employed. Doelger and Prescott advocate 26 to 28°C. as the m temperature range. They state that the "amount of citric acid d will be on a rising scale as the temperatures are increased from 3°C." and that at 30°C. or above "citric acid production will e and a greater proportion of the titrable acidity will be due to nation of oxalic acid." Table 148 shows the effect of the tempera- incubation on titrable acidity and the quantity of citric acid Standard medium was used in the experiment.

0-ml. flasks were placed 75-ml. portions, and all were inoculated e same 10-day-old culture of mold spores (*A. niger*).

a 10-day period of incubation, samples from each flask (10 at mperature) were analyzed, with the results shown in Table 148.

18. EFFECT OF TEMPERATURE OF INCUBATION ON TITRABLE ACIDITY AND CITRIC ACID PRODUCED *

| of on, | Titrable acidity, normality | Citric acid produced | | Evaporation of medium, per cent |
|---|---|---|---|---|
| | | Per flask fermentation, g. | Per 100 g. sugar, g. | |
| | 1.0024 | 3.37 | 32 | 24 |
| | 1.0535 | 3.55 | 34 | 27 |
| | 1.1187 | 3.96 | 38 | 26 |
| | 1.1564 | 3.88 | 37 | 30 |
| | 1.1045 | 2.87 | 27 | 36 |

. Doelger and S. C. Prescott, *Ind. Eng. Chem.*, 26: 1142 (1934). Copyright he American Chemical Society and reprinted by permission of the copyright

**tion of Fermentation Period.** In the production of citric acid shallow-pan method, the fermentation is usually complete in days.

**s.** On the basis of the sugar consumed, a maximum yield of cent of citric acid was obtained from glucose by Wells, Moyer, y. Carbon balances were prepared to show exactly how the car- used during the fermentations. Clutterbuck and his associates cured yields as high as 87 per cent on a semicommercial scale. mounting to about 100 per cent on the basis of the sugar con- have been reported by Butkewitsch and Gaewskaya. Usually, however, about 60 per cent of the weight of the sugar used in the may be recovered as citric acid.

**Recovery of Citric Acid.** At the completion of the fermenta solution is drained off and the mat is pressed to remove any acid c in it. Calcium citrate is then precipitated from a hot neutral By treating the precipitate with an equivalent of sulfuric acid, t acid is liberated and is recovered by separating it from the sulfate.

The unconverted sugar may be fermented by yeasts and the cit crystallized directly, in an alternate method.

**Cultural Methods.** The successive transfer of spores from one medium to another of the same uniform composition may stimu mold to produce large yields of citric acid. Doelger and Presc demonstrated the effect of 18 successive transfers on titrable Twelve 250-ml. flasks, each containing 75 ml. of the standard (14 per cent sucrose) adjusted to a pH of 2.00, were inoculated w spores of a strain of *A. niger* from a single culture and incubate temperature of 26°C. for 10 days. Twelve new flasks were ino from the spores that appeared on the mycelial mats of the first flasks. This process was repeated at intervals of 10 days for a months. Results of this experiment are shown in Table 149.

Table 149. Average Titrable Acidity Produced with 18 Successive Spore Tr

| No. of inoculation series | Average titrable acidity, normality | Increase in titrable acidity from low point, normality | Average spo mat, per c |
|---|---|---|---|
| 1 | 0.4544 | ...... | 10.1 |
| 2 | 0.3438 | ...... | 53.2 |
| 3 | 0.2844 | 0.0000 | 32.7 |
| 4 | 0.3298 | 0.0454 | 14.5 |
| 5 | 0.3430 | 0.0586 | 9.5 |
| 6 | 0.3780 | 0.0936 | 8.0 |
| 7 | 0.3250 | 0.0406 | 6.7 |
| 8 | 0.4703 | 0.1859 | 5.0 |
| 9 | 0.4308 | 0.1464 | 5.5 |
| 10 | 0.4801 | 0.1956 | 7.1 |
| 11 | 0.3595 | 0.0751 | 32.2 |
| 12 | 0.5507 | 0.2663 | 3.3 |
| 13 | 0.5213 | 0.2369 | 3.1 |
| 14 | 0.5718 | 0.2774 | 3.3 |
| 15 | 0.4717 | 0.1873 | 1.4 |
| 16 | 0.6959 | 0.4115 | 1.6 |
| 17 | 0.6232 | 0.3388 | 3.3 |
| 18 | 0.8116 | 0.5272 | 1.3 |

The figures contained in Table 149 indicate a general increase level of titrable acidity with successive transfers. The drop in ti acidity after the first three transfers may be ascribed to the fact th mold had been grown previously in a 10 per cent sucrose solutio d time to become adjusted to the new concentration. After pro- a titrable acidity approximately equivalent to a 1.2 *N* solution, usually no further increase in the titrable acidity.

right-hand column of the table shows the relation of sporulation ls. The term "average spores on mat" refers to the approximate age of the surface covered by spores. Those fermentations in igh yields of citric acid were obtained showed only a few spores a complete absence of spores. Thus sporulation may be used as s of judging the efficiency of a fermentation.

eeding only one-fourth to one-half of the surface area of the n with spores, Doelger and Prescott obtained the best results. n sprouting of the spores is prevented if the entire surface is seeded, e result that unsprouted spores become embedded in the mycelial is believed that the unsprouted spores may exert a toxic effect citric acid production.

a detailed discussion of the technique of the fermentation, the s referred to the publications of Currie, Doelger and Prescott, and (see the bibliography at the end of this chapter).

**acteristics of the Fermentation.** Sterile nutrient sugar solutions low pans are inoculated with mold spores and incubated at the vorable temperature for fermentation. The spores commence to fter a few hours, and within 2 to 5 days the surface of the medium ed by a firm, mycelial mat. With the formation of the mycelial ric acid production proceeds at a rapid rate, and the fermentation ly completed in 7 to 10 days.

e 94 shows curves for titrable acidity, pH, and weight of the mat hes that were allowed to incubate for 20 days at 24°C.

about 90 per cent of the sucrose originally present in the medium n converted to other products, the rate of increase of titrable diminishes. Likewise when the concentration of citric acid is than approximately 7 per cent, it retards the rate of increase of acidity. In a normal fermentation, the titrable acidity increases e ninth or tenth day, at which time there will be 7 to 8 per cent acid and less than 1 per cent of oxalic acid. (An 8 per cent citric centration is equivalent to a 1.2 *N* solution.) Citric acid is broken less it is recovered within a reasonable time after being produced.

**uction from Cane Molasses.** Perlman, Kita, and Peterson (1946) citric acid production from cane molasses. They found that yields ins of *A. niger* from untreated solutions of molasses were low in ison with those obtained from synthetic and beet-molasses media. nthetic medium contained 140 g. of purified sucrose, 2.25 g. of $_3$, 1.00 g. of $KH_2PO_4$, 0.25 g. of $MgSO_4 \cdot 7H_2O$, 0.1 mg. of Fe (as $SO_4)_2 \cdot 12H_2O)$, HCl to a pH of 2.3, and distilled water to 1 liter. t-molasses medium contained 280 g. of beet molasses diluted er with distilled water. This was treated by adding 1.2 g. of $N)_6 \cdot 4H_2O$, 2 ml. of 1 *N* $H_2SO_4$, and 10 g. of filter cell and per- precipitation to take place over a period of 2 days at 10°C. The tant solution had a pH of 6.5.

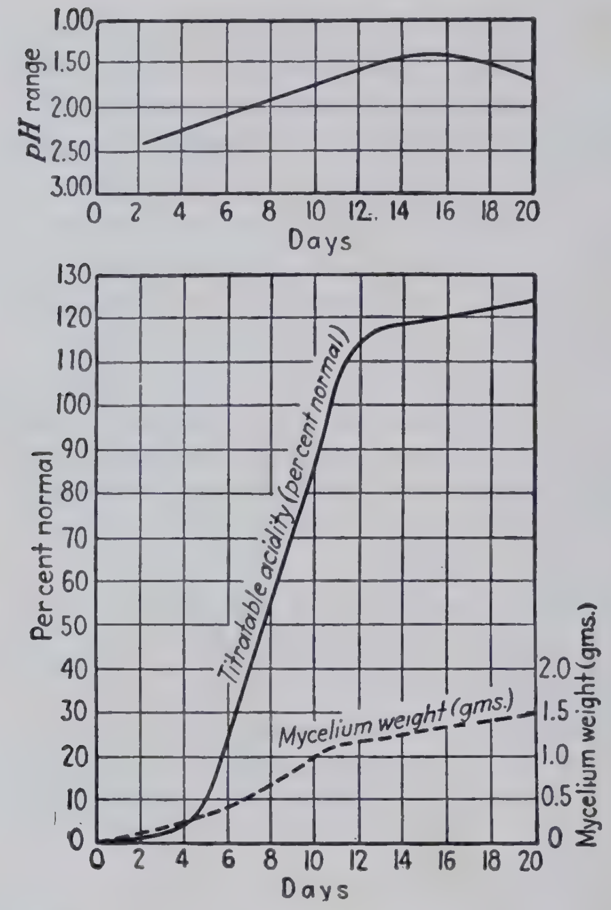


FIG. 94. Rate of increase in titrable acidity, weight of mat, and pH range in fermentations. [*From W. P. Doelger and S. C. Prescott, Ind. Eng. Chem.,* 26: (1934). *Copyright* 1934 *by the American Chemical Society and reprinted by pe sion of the copyright owner.*]

Replacement of a part of the synthetic, or beet-molasses, medium a Cuban high-test-molasses medium (180 g. of molasses, 2.25 $NH_4NO_3$, 1.0 g. of $KH_2PO_4$, 0.25 g. of $MgSO_4 \cdot 7H_2O$, and distilled to 1 liter), or the addition of small amounts of ash from the Cuban test molasses to these media, resulted in lower yields of citric acid. ashes from other samples of cane molasses and from untreated molasses gave similar results. However, the ash from ferrocyanide-tre beet molasses did not cause a reduction in yields when added to the thetic and beet-molasses media. Thus, Perlman and his associates cluded that the inorganic materials found in molasses were respon for at least part of the inhibitory effect on citric acid production.

Treatment of the molasses with cation-exchange material or with po sium ferrocyanide reduced the amount of ash and resulted in impr yields in most cases.

Lamb (1936); Das Gupta, Saha, and Guha (1940); and Chatt (1942) also have reported on studies made on the use of molasse citric acid production.

A considerable amount of research on the production of citric acid cane molasses by the surface-culture method has been carried o South Africa. Oxford (1949) discussed the difficulties inherent in

ation of cane molasses and the lines of research initiated by his
ory. Oxford, Sephton, and Van der Westhuizen (1949*a*) found that
er of strains of *A. niger* and *A. wentii* were able to ferment Natal
lasses satisfactorily to citric acid. The optimum concentration of
s appeared to be about 33 per cent, 25 per cent being too low and
ent being too high. The natural initial pH of the molasses mash,
as 5 to 5.5, appeared to be best for the fermentation. A tempera-
between 20 and 28°C. was satisfactory, while 32.5°C. was too high.
nfluence of inorganic anions on the fermentation was studied by
Sephton, and Van der Westhuizen (1949*b*), who found that rela-
igh concentrations (about half normal) of the chlorides of magne-
tassium, and sodium suppressed the formation of oxalic acid to a
reater extent than that of citric acid. It was possible to inhibit
cid formation entirely, or nearly so, by the use of sufficient chlo-
he lower the initial pH and the phosphate concentration, the
the concentration of chloride that was required to suppress oxalic
mation. The corresponding sulfates in equivalent concentrations
very little effect on oxalic acid formation, while the nitrates
d to exert an effect intermediate between that of the chlorides and
ates.

**uction from Beet Molasses.** The production of citric acid from
lasses had been investigated by Roberts and Murphy (1944); by
lt, Dorrell, and Baldwin (1946); and by others.

e laboratory method of Roberts and Murphy beet molasses con-
calcium carbonate is impregnated on sphagnum moss. The soaked
m moss is distributed in a layer 1 to 2 in. deep, inoculated with
res of *A. niger*, and incubated at 25°C. Yields of 25 to 30 g. of
citrate from 200 g. of molasses have been obtained in 2 days.
gluconate is also produced.

olds used by Gerhardt and associates were two strains of *A. niger:*
2 (No. 67 of Wells, Moyer, and May, 1936), and strain 72 (Ameri-
e Culture Collection No. 1015). These were selected from a
20 on the basis of their superior fermentation ability. Spores
ulation purposes were grown in bottle plates containing sucrose
agar. After 6 to 25 days, spore suspensions were prepared from
ures and were standardized to contain about 50 million spores
Approximately 1 ml. of suspension was used to inoculate a surface
50 sq. cm. and 20 ml. for 5 liters of medium (5 cm. deep) in an
m pan measuring 36 by 25.5 by 7.5 cm.

ugh four samples of Straighthous beet molasses from different
s were treated with potassium ferrocyanide, optimum conditions.
ere as follows, were determined for only one sample: A quantity
. of beet molasses, partially diluted with water, was treated with
f potassium ferrocyanide (in solution). The mixture was made
liter with distilled water. Then 10 g. of diatomaceous earth was
nd mixed in uniformly. The mixture was permitted to stand over-
about 6°C. in a graduated cylinder or similar container. The
medium was dispensed in the fermentation containers (6-oz.

bottles, glass tumblers, or shallow aluminum pans) and autocl 120°C. for 15 min. The sugar concentration of the final mediu about 15 per cent.

Effective treatment of beet molasses with potassium ferrocyanide on the yields of citric acid subsequently obtained from the mediu found to depend upon the concentration of ferrocyanide used, the the molasses, and the conditions of sterilization. Relatively small tions from the optimum concentration of potassium ferrocyanid found to result in reduced yields from the one sample of molasses However, the yields of citric acid obtained from beet molasses t with potassium ferrocyanide were nearly equal, and small variation the optimum concentrations did not appreciably influence the yields optimum pH for the ferrocyanide treatment of beet molasses was ab regardless of whether the adjustment of pH was made before or precipitation or after sterilization. Adjustment of the pH was unn sary in the case of some samples of beet molasses. The yields of acid were higher for one sample of beet molasses when the medium sterilized, and for three other samples when the medium was not steri

The media were incubated at 30°C. for 10 days after inoculation.

The yields of citric acid obtained from beet molasses in the sh aluminum pans (covered loosely during fermentation with sheet minum) were 45 to 50 per cent based on the available sugar.

**Citric Acid by the Cahn Method.** A rather unusual method of acid production was advocated by Cahn in 1935. Solid material, as cane or beet pulp, is impregnated with sugar solutions—sucros molasses. The unsterilized mass is inoculated with a mold culture, age of which is said to be relatively unimportant, and fermentation ceeds rapidly over the relatively large surface at a temperature of 2 35°C. The fermentation is usually complete in 4 days or less, at w time a yield of 55 per cent citric acid on the basis of the sucrose origin present, or 45 per cent acid, calculated on the basis of the sugar origin present in the molasses, may be expected. One pound of citric acid be secured from 5.7 lb. of molasses and 2.75 lb. of beet pulp (beet may be used only once, while cane pulp may be used more than once)

**Production of Citric Acid by a Mucor.** Solutions of molasses and have been fermented by a species of *Mucor* with the production of c and oxalic acids (Das Gupta et al., 1938). By controlling the condit of the fermentation, citric acid may be obtained with little or no ox acid. A molasses mash containing 1 per cent added ammonium sulf adjusted to a pH of 4, and incubated at a temperature of 28°C. f period of 7 days yielded 33 per cent of citric acid on the basis of sugar.

**The Production of Citric Acid on an Industrial Scale.** The earl attempt to produce citric acid by fermentation on an industrial scale made in Germany around the beginning of the twentieth century. many difficulties encountered made it impossible to meet the competi offered by the citrus-fruit industry, and the attempt was abandoned.

It is believed that some citric acid was produced by fermentation o

cial basis in the United States in the year 1919, but it was not
e year 1923 that appreciable quantities were manufactured by
ological process, the first factory in the world for the commercial
ion of citric acid by fermentation being erected in New York
ir.

## DUCTION OF CITRIC ACID BY SUBMERGED-CULTURE METHODS

efforts to produce citric acid by submerged-culture methods
in failure. Yields produced by this method were lower than those
l by surface-culture methods, and occasionally gluconic acid was
d at the expense of citric acid.

ıer (1912) endeavored to produce citric acid from a nutrient
medium containing chalk by passing sterile air through it. Citric
s produced as calcium citrate, but the conversion was low. Simi-
lts were obtained by Elving (1918–1919), who caused the mold to
submerged cultures by means of aeration. In 1926, Bleyer reported
ocess in which citric acid was produced in vats supplied with air
asional mechanical agitation. Schreyer (1928) discovered that
ı and continuous mechanical agitation increased the total pro-
of acid by *Aspergillus fumaricus* over that of controls in which
ace-culture method was used but that gluconic acid and not citric
oduction was increased. Results obtained by Thies (1930) were
to those of Schreyer, for he found that aeration of the medium
erile oxygen increased gluconic but not citric acid production.
g (1930) experimented in the production of citric acid from
by *A. japonicus* in submerged culture using aeration. Although
lds were obtained by this method, they were not as high as those
l by the older method of culture in shallow layers without aera-
rquin (1938) discovered that the addition of solutions of glucose
se to the preformed mycelium of *A. niger* resulted in gluconic acid
on. However, some citric acid was produced when the solution
d zinc sulfate, potassium chloride, and ammonium chloride and
low pH.

ssful production of citric acid on a laboratory or pilot-plant scale
ı reported by a number of investigators (refer to Table 150).

**Factors Affecting Production.** There are a number of factors
ect citric acid production by submerged-culture methods. Some of
ill be discussed now, others later.

ıs of molds differ markedly in their ability to produce citric acid
submerged-culture method. Perlman confirmed this by testing
ns of *A. niger* for their ability to produce citric acid on five differ-
ia: glucose salts, sucrose salts, maltose sirup, beet molasses, and
nide-treated beet-molasses media. The facts he derived empha-
need for selecting strains carefully.

ported that young spores are better than old ones for producing
id and that the temperature of storage of spores (on agar slants)

Table 150. Some Literature Citations concerning the Production of C
by Laboratory, Pilot-plant, or Commercial-scale Methods

Wehmer, C.: *Chem. Ztg.*, **36**: 1106 (1912).
Elving: *Overs. Finska Soc. Ford. Math. Naturw.*, **61**(15) (1918–1919).
Bleyer, B.: German Patent 434729, Oct. 6, 1926.
Amelung, H.: *Hoppe-Seyler's Zeit. physiol. Chem.*, **166**: 161 (1927); *C*
**54**: 118 (1930).
Kluyver, A. J., and L. H. C. Perquin: *Biochem. Zeit.*, **266**: 68 (1937).
Perquin, L. H. C.: Thesis, Delft, 1938.
Schreyer: *Biochem. Zeit.*, **202**: 131 (1938).
Szücs, J.: U.S. Patent 2,353,771, July 18, 1944.
Karow, E. O.: Thesis, Rutgers University, New Brunswick, N.J., 1942.
——— and S. A. Waksman: *Ind. Eng. Chem.*, **39**: 821 (1947); U.S. Patent
Perlman, D.: *Econ. Botany*, **3**: 369 (1949).
Schweiger, L. B., and R. L. Snell: U.S. Patent 2,476,159, July 12, 1949.
Foster, J. W.: "Chemical Activities of Fungi," Academic Press, Inc., N
1949.
Prescott, S. C., and C. G. Dunn: "Industrial Microbiology," 2d ed., McC
Book Company, Inc., New York, 1949.
Snell, R. L., and L. B. Schweiger: U.S. Patent 2,472,667, Dec. 27, 1949.
Woodward, J. C., R. L. Snell, and R. S. Nichols: U.S. Patent 2,492,673, Dec.
Martin, S. M., and W. R. Waters: *Ind. Eng. Chem.*, **44**: 2229 (1952).
Clement, M. T.: *Can. Jour. Technol.*, **30**: 82 (1952).
Buelow, G. H., and M. J. Johnson: *Ind. Eng. Chem.*, **44**: 2945 (1952).
Moyer, A. J.: *Appl. Microbiol.*, **1**(1): 1 (1953).
Martin, S. M.: *Can. Jour. Microbiol.*, **1**: 644 (1955).
Steel, R., C. P. Lentz, and S. M. Martin: *Can. Jour. Microbiol.*, **1**: 299 (1955)
Gardner, J. F., J. Valerie-James, and S. D. Rubbo: *Jour. gen. Microbiol.*,
(1956).

has a big effect on their ability to produce citric acid. Of the te
tures tested, 0 to 29°C., the range of 0 to 5°C. is best for storage.
rioration in ability to produce citric acid is most rapid at 29°C.

The influence of the composition of the sporulation medium o
acid production by *A. niger* (strain 72–4) has been studied by S
Johnson (1947). They made a number of significant observatio
conclusions. The effect of increasing the concentrations of zinc,
nium nitrate, and potassium dihydrogen phosphate in the basic s
tion medium was to decrease the rate of spore formation. Increas
concentrations of manganese to 9.3 mg. per liter and malt extract
g. per liter favored abundant spore formation within 48 hr. instead
usual 68 hr.

However, the addition of 9.3 mg. per liter of manganese to the
lation medium reduced the yield of citric acid from the fermen
medium, whether used alone or in combination with traces of zinc,
and/or iron. It was shown that the amount of manganese carried
the sporulation medium with washed spores was sufficient in amo
reduce the yields of citric acid from the fermentation medium, and
mere 3 μg. of manganese per liter in the latter medium lowered
appreciably.

Trommer malt extract, but not its ash, when added to the sporu
medium reduced citric acid production in submerged cultures.

malt extract nor manganese had any appreciable effect on citric acid luction by the surface-culture method, however.

'erlman also observed that when zinc and/or manganese salts were ed to a purified agar-sucrose-salts medium, the spores produced eon often produced low yields of citric acid. However, this varied siderably with the strain of mold used.

emperature of incubation plays an important part in the production accumulation of citric acid. As a rule most strains produce considermore citric acid at 26 to 30°C. than at 39°C. Perlman also showed there may be a loss in the citric acid content of flasks if these are ed at a higher temperature following initial incubation at a lower erature.

ıe influence of heavy metals on surface-culture fermentations has idy been referred to. Such ions also have an effect on citric acid uction by the submerged-culture method, depending on the strain of used and other factors. There appears to be an optimum concenon for each ion. The low yields of citric acid obtained from some ical grades of sugar and molasses are undoubtedly due, in part at to an inhibiting concentration of certain ions. Sjolander (1945) has n that the addition of the ash of these carbohydrate sources to a ıetic medium will result in a reduced yield of citric acid.

**oduction with Aspergillus wentii.** This fermentation has been ibed in a series of publications by Karow and Waksman. The folg discussion is based on these reports.

e process used by Karow and Waksman is singular in several ts: (1) the mold, (2) the use of a special medium for the growth and ioning of the mold, (3) the aeration of the medium with oxygen or der pressure together with mechanical agitation, (4) the use of a ement medium for the production of citric acid with preformed cell ial, and (5) the partial neutralization of the citric acid in the ement medium to increase the yield of citric acid. Production by ethod may be divided into two phases—a growth and a fermentaıase.

*Mold.* A selected strain of *A. wentii* produced the highest yields c acid by submerged growth in the researches carried out by Karow aksman. *A. niger* 2.3, which was used by Perquin, was found to be r to *A. wentii.*

colonies produced from the mold spores of *A. wentii* under subd-growth conditions are generally globular in appearance but occay may be stringy. Superior results in the production of citric acid been achieved through the use of cultures containing globular s. Stringy colonies usually result from too rapid growth brought y the inclusion in the medium of substances that are particularly ole to growth, such as high nitrogen and iron.

*ohydrate Sources.* Various sources, such as sucrose, glucose, corn ıydrol, sugar-cane juice, raw sugar, and molasses, are satisfactory ic acid production. Sucrose, in particular, and glucose are good . Raw sugar, though good, is inferior to sucrose. Purified cane molasses is suitable, the yields from some types being as high obtained from pure cane sugar.

Ion-exchange resins, activated carbon, bone char, and bauxite used to purify the molasses. The treatment of molasses with a tion of bone char, Suchar CSP, and Amberlites (ion-exchang yielded excellent results.

*The Growth Medium.* Karow found that the following medi best for growing *A. wentii* and citric acid production:

| | |
|---|---|
| Sucrose | 150 g. |
| Urea | 1.0 g. |
| $MgSO_4 \cdot 7H_2O$ | 0.5 g. |
| $KH_2PO_4$ | 0.08 g. |
| KCl | 0.15 g. |
| $MnSO_4 \cdot 4H_20$ | 0.02 g. |
| $ZnSO_4 \cdot 7H_2O$ | 0.01 g. |
| Water (distilled) to | 1,000 ml. |
| pH adjusted to 2 with HCl | |

The use of higher concentrations of urea resulted in increased A high nitrogen content in general increases growth and the consu of sugar but tends to decrease the amount of citric acid produced.

The amount of magnesium sulfate used may be varied appr without seriously affecting growth or citric acid production, as l sufficient is furnished to supply basal requirements. Effects caus the use of larger quantities of magnesium sulfate usually appeared in the fermentation and disappeared later.

The optimum concentration of manganese sulfate appeared to be per liter. Larger concentrations of this salt did not stimulate citri production. It is possible that the manganese may act by help maintain a favorable oxidation-reduction potential.

Zinc sulfate in the proportion of 10 mg. per liter increased the of citric acid when manganese sulfate was also present in a concent of 20 mg. per liter. Without manganese sulfate, zinc sulfate dec the yields of citric acid. Manganese thus tends to antagonize the i tory effects of zinc.

Other effects of salts on citric acid production by *A. wentii* have found. For example, the omission of potassium chloride from the medium appeared to result in decreased acid production.

Accessory growth factors had no apparent effect on the producti citric acid by *A. wentii.* Thiamin chloride, pantothenic acid, ribof pyridoxine, nicotinic acid, ascorbic acid, and *p*-aminobenzoic acid, alone, and in combination, were used in the basal medium without on citric acid production. Corn-steep liquor increased the rate amount of growth and total titrable acidity but not the citric acid Yeast extract also increased the rate and amount of mycelial growth sugar consumption, but did not produce appreciable increases in acid accumulation.

*Inoculation of the Medium.* Karow states that citric acid produ by *A. wentii* from the growth medium is inversely proportional of the inoculum. For 100-ml. amounts of medium, an inoculum con- ing 0.25 to 1 ml. of spores in suspension is adequate. Amounts larger 1 ml. result in lower yields of citric acid.

fter the medium is inoculated it should be permitted to stand for 12 8 hr. before agitation and oxygenation are commenced; otherwise the l of citric acid is decreased.

*ffect of Oxygen.* Oxygen has a profound effect on the production of e acid by *A. wentii.* Best yields are obtained when oxygen is passed ugh the submerged cultures. Aerated cultures produce larger yields tric acid than unaerated cultures, but yields from aerated cultures decidedly lower than those from oxygenated cultures. The use of en results in greater sugar consumption and development of myce- . Oxygen, pure or combined with nitrogen, is essential for the produc- of citric acid by *A. wentii* and also for the growth of the mold in ierged cultures.

*fect of Carbon Dioxide.* Although it has been shown by Foster, on, Ruben, and Kamen (1941) that carbon dioxide participates in the iesis of citric acid by *A. niger*, the addition of this gas to submerged res of *A. wentii* at pH 2 does not result in increased yields of citric Actually 5 and 15 per cent concentrations of carbon dioxide inhibit roduction of this acid. The supply of carbon dioxide also results ower consumption of sugar and decreased growth of cell material.

*e Replacement Medium.* The replacement medium was devised in to study the effect of various minerals on preformed cell material. atter was produced by inoculating a sterile nutrient solution (de- d above) with a heavy spore suspension of *A. wentii*, incubating C., and aerating with sterile air. Cell material available after 4 incubation was used in the study, since it was discovered that young ium was more efficient than old.

replacement medium, selected after considerable research, had the ing composition:

| | |
|---|---|
| Sucrose | 150 g. |
| Urea | 0.5 g. |
| $KH_2PO_4$ | 0.05 g. |
| KCl | 0.15 g. |
| $MnSO \cdot 4H_2O$ | 0.02 g. |
| $ZnSO_4 \cdot 7H_2O$ | 0.01 g. |
| Water to | 1,000 ml. |

nt nutrient salts were added to the medium to maintain the mold ictive enzymic condition and to produce a slight increase in growth. en (from urea) and phosphate (from $KH_2PO_4$) stimulated the tion of citric acid. Magnesium sulfate had an inhibiting effect and as not added to the medium.

*t of Partial Neutralization of Replacement Medium.* Partial neu- ion of the acid produced by *A. wentii* in the replacement medium use of calcium carbonate leads to increased production of citric When the acid is completely neutralized, oxalic rather than citric formed. Neutralization of the acid to a pH higher than the range of 3 to 3.5 leads to oxalic acid production, as does neutraliz sodium, potassium, or ammonium hydroxide. As much as a th acid can be neutralized with $CaCO_3$ without producing a pI as 3.5.

*Pilot-plant Production.* Karow carried out an experiment usi bottle. Five liters of the growth medium was placed in it and i with the spores of *A. wentii.* The bottle was caused to rotate o at the rate of 21 r.p.m., and 6 to 8 liters of oxygen per hour wa to the medium. After the tenth day, acid production dropped The cell material was filtered and washed with sterile distill Five liters of the replacement medium was added, and fermenta tinued for 10 days under conditions similar to the preceding. 4 liters of the growth solution, 135.6 g. of anhydrous citric acid w ered; from 3.7 liters of the replacement solution, 198.7 g.

For further details on this process, the reader is referred to th by Karow and Waksman cited herein.

**Production by the Szücs Method.** In this method for produci acid by submerged fermentation, the mold is first cultured in a solution. The latter may be inoculated with the spores, pregen spores, mycelium, or comminuted mycelial mat of *A. niger* or ot species that has the ability to produce citric acid from carbohyd surface fermentation. Finely dispersed, sterile, oxygen-containin passed through the medium, which is also stirred. After de sufficiently in the growth solution, the mycelium is separated centrifuging, decanting, or filtration and placed in a second known as the "fermentation solution." This solution contains a hydrate, such as sucrose, fructose, glucose, purified molasses, corn corn sugar, and nutrient salts but contains no assimilable pho compounds. (The presence of a small quantity of phosphorus con will retard but not prevent the formation of citric acid.) The fe tion solution is agitated and stirred. An oxygen-containing gas (u oxygen or mixtures of oxygen with nitrogen or air) is passed thro solution in a finely dispersed state, with or without pressure. Fo ple, oxygen or a mixture of oxygen and air containing 40 to 50 by volume of oxygen, may be passed through the solution at th atmospheric pressure or under pressures of 1 to 4 atmospheres. carbon, ascorbic acid, glutathione, or other accelerators that act as carriers may be added to the solution. Calcium carbonate or som suitable neutralizing agent may be added to the fermentation before or during the fermentation for the purpose of neutralizing th acid. The fermentation may be carried out in the temperature r 18 to 28°C. However, a temperature of 25°C. is preferred by Szücs the fermentation is completed, the mycelium may be removed fr solution and used in one or more additional submerged fermentati

An example of this fermentation follows: A growth solution contained 25 to 50 g. of sucrose, 2.25 g. of $NH_4NO_3$, 0.3 g. of K 0.25 g. of $MgSO_4 \cdot 7H_2O$, and 10 ml. of $N/1$ HCl (pH 2) in 1 lit sterilized and inoculated with the spores of *A. niger* in aqueous

n. The solution was stirred, and sterile air in a finely dispersed condi- a was passed through it. After growth for 3 to 4 days at 25°C., the celium was separated from the growth solution and washed with dis- ed water. The washed mycelium (5 parts by weight in terms of dry celium for 1,000 parts by weight of the fermentation solution) was n placed into the fermentation solution, which contained 200 g. of rose, 1.1 g. of $NH_4NO_3$, 0.15 g. of KCl, 0.25 g. of $MgSO_4 \cdot 7H_2O$, and nl. of *N*/1 HCl (pH 1.91) in 1 liter. The fermentation solution was ated vigorously with a rotary stirrer revolving at 300 r.p.m., and ly dispersed oxygen was passed through it (100 ml. through 2 liters of tion per minute). The temperature was maintained at 25°C. In about ys, a yield of 70 to 75 per cent of citric acid, on the basis of the sugar umed by the mold, was secured.

**roduction by the Method of Shu and Johnson.** According to Shu Johnson, yields of 72 g. of anhydrous citric acid may be obtained from g. of sucrose in 9 days by a submerged-fermentation process, while a strain of *A. niger* derived from A.T.C.C. No. 1015.

e organism was carried in soil cultures. Before use it was transferred on sugar agar slants and then cultured on a sporulation medium lium A in Table 151) for 3 to 5 days. The suspension prepared from pores was used as the inoculum.

TABLE 151. COMPOSITION OF MEDIA *

| Constituents | Medium A, wt./liter | Medium B, wt./liter |
|---|---|---|
| Domino sucrose, g. | 140 | 140 |
| Bacto agar, g. | 20 | |
| $KH_2PO_4$, g. | 1.0 | 2.5 |
| $MgSO_4 \cdot 7H_2O$, g. | 0.25 | 0.25 |
| $NH_4NO_3$, g. | 2.5 | 2.5 |
| HCl to pH 3.8 | | |
| Trace metal †: | | |
| $Cu^{++}$, mg. | 0.48 | 0.06 |
| $Zn^{++}$, mg. | 3.8 | 0.25 |
| $Fe^{+++}$, mg. | 2.2 | 1.3 |
| $Mn^{++}$, mg.‡ | < 1.0 | < 1.0 |

hu and M. J. Johnson, *Ind. Eng. Chem.*, 40(7): 1202 (1948). Copyright 1948 American Chemical Society and reprinted by permission of the copyright

ed quantities of metals include amounts present as impurities in other con- s of the medium. Media were sterilized at 120°C. for 15 min.

importance of low manganese concentrations in both sporulation and fer- n media for submerged citric acid production is demonstrated in work elsewhere.

basal medium was prepared as indicated in Table 151, adjusted to f 3.8 with hydrochloric acid, and distributed in 50-ml. amounts in Erlenmeyer flasks. These were inoculated with spore suspensions.

The flasks were then agitated at 270 r.p.m. on a rotary shaker i manner that each described a horizontal circle 1 in. in diamet incubation temperature was 25°C.

A number of factors were found to be important in obtaini yields of citric acid. Sucrose concentrations of 140 to 264 g. per li optimum for citric acid production on the basis of sugar utiliz yields were obtained with low sugar concentrations. It was nece keep the manganese content of both the sporulation and the ferm media low to obtain good yields. Magnesium sulfate in concen below 0.5 g. per liter resulted in lower yields, while amounts fro 2 g. per liter did not affect the conversion efficiency. Iron was i directly with the growth of the mycelium and with the citric aci A concentration of 1 mg. per liter was optimum. At too low con tions of iron the utilization of sugar was poor. Large concent favored heavy growth of the mycelium, which was inclined to r smaller yields. The phosphate ion appeared to be concerned wit acid production, in addition to its effects as a buffer and food cons The optimum initial pH range was 2.2 to 4.2. At too low init levels, growth and acid production were retarded. Maximum yiel obtained in 8 days with the aeration and agitation provided by a shaker and in 11 days by a reciprocating type of shaker.

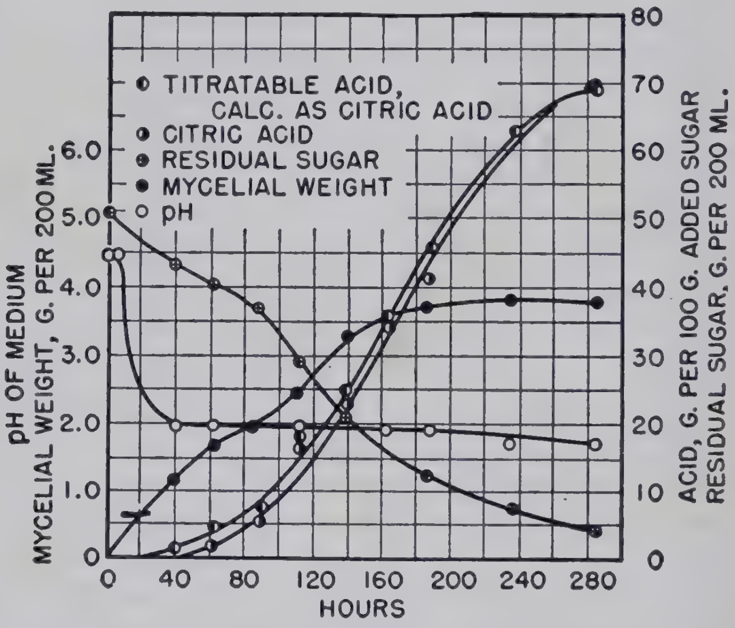


FIG. 95. Chemical changes during fermentation. [*Courtesy of P. Shu and M. J son, Ind. Eng. Chem.,* 40(7): 1202 (1948). *Copyright* 1948 *by the American Ch Society and reprinted by permission of the copyright owner.*]

The chemical changes occurring during a fermentation of the medium (medium B in Table 151), containing 25 per cent sugar and initial pH of 4.5, are shown in Fig. 95.

**Effect of Ferrocyanide on Growth and Acid Production.** The potassium ferrocyanide for the treatment of molasses mashes to im the yield of citric acid has been reported by Gerhardt et al. (1 ıller (1946), Bernhauer et al. (1949), Clement (1952), Martin and ters (1952), Steel et al. (1954, 1955), Martin (1955), and others.

'he amount of ferrocyanide used depends upon the composition of the 'asses primarily. Clement added 0.6 to 0.8 g. per liter of potassium ocyanide to the sterilized beet-molasses medium while the latter was Steel et al. (1955) adjusted the beet molasses with tap water to a centration (weight per volume basis) of 24 per cent and the pH with rochloric acid to 6. The mashes were then sterilized for 45 min. at .s.i.g. Ferrocyanide was added immediately after sterilization. Levels .5 to 0.6 g. per liter of $K_4Fe(CN)_6 \cdot 3H_2O$ were found to be optimal, a range of 0.4 to 0.8 g. per liter generally resulted in good yields of c acid.

artin (1955) studied the effect of ferrocyanide on growth and acid uction by *A. niger* (NRC A1-233). He found that a concentration of than 1 p.p.m. of ferrocyanide inhibited the growth of the mold in a le synthetic medium and that the inhibition could be reversed by ng increased amounts of iron and zinc to the medium. Ferrocyanide less toxic to the mold at a reaction close to neutrality. However, acid uction, which was not inhibited materially at low concentrations of 'errocyanide, increased the toxicity of the latter to the mold and ually stopped its growth. Neither inhibition of the growth of *ger* nor acid production was caused by the release of cyanide from cyanide. Martin postulated that ferrocyanide performed at least two rtant functions during the treatment of media for increased yields ric acid: one was to remove the trace metals, which stimulate growth ot citric acid formation; the other was to act as a direct growth tor.

**ect of Alcohols.** Moyer (1953*a*) studied the effects of alcohols on acid production. Methyl and ethyl alcohols in concentrations of per cent and isopropyl alcohol in a concentration of up to 2 per cent sed the production of citric acid from glucose by *A. niger* NRRL 567 face cultures. At a concentration of 3 per cent, isopropyl alcohol xic. There was also a reduction in mycelial growth associated with of the alcohols. It was found that ethyl alcohol was used to some as a carbon source by the mold.

oncentration of 3 per cent of added methanol increased the yields ic acid from *A. niger* NRRL strains 67, 321, 326, 335, 340, 350, 367, 67, 604, and 1736 and *A. wentii* NRRL 2001 and also inhibited ation in the production medium in surface culture. Some strains ds possessed a low tolerance for methanol, however, and produced ightly increased yields.

rance of the mold to methanol was found to be associated with the the medium. At a pH of 2.48 and above, Moyer observed that a ent concentration of added methanol was best. However, at a pH 2 per cent of added methanol was optimum.

pH of 6.5, gluconic acid is generally produced by *A. niger* in the e of calcium carbonate. When 1 to 2 per cent of methanol was o the production medium, a mixture of gluconic and citric acids resulted; but when 3 to 4 per cent of methanol was added, high citric acid were formed.

The effect of the amount of inoculum and methanol concentr citric acid production by *A. niger* NRRL 372 in submerged cu shown in Table 152. It will be observed that methanol concentra 1.5 and 2 per cent were more effective in forming increased yields acid when the inoculum was 1 per cent than when it was 2 or 3 p The highest yield of citric acid was obtained with 3 per cent of m and 3 per cent of inoculum at an initial pH of 4.05.

Acidification of the production medium with 0.5 *N* citric acid to initial pH values of 2.35 or 2.6 resulted in increased citric acid y submerged culture in the presence of 2 to 2.5 per cent methanol.

Corn starch, ground corn, wheat starch, high-test beet and bla molasses, and commercial glucose were fermented to citric acid presence of a concentration of 1 to 4 per cent by volume of methanol (Moyer, 1953*b*).

Apparently one of the effects of the alcohols is to increase the to levels of manganese, iron, and zinc considerably above those need the growth of mycelium, permitting the use of crude carbohydrate s such as blackstrap molasses, and media better balanced nutriti according to Moyer.

**Effects of Phosphate.** Assimilable phosphates are essential f growth of molds. They appear also to exert profound effects o amounts of some of the acids formed in the citric acid fermentation

Szücs (1944) was first to show that the presence of a small amo assimilable phosphorus compounds in the aqueous fermentation so retarded but did not prevent citric acid formation by *A. niger* submerged-culture conditions. Citric acid production started aft available phosphorus compounds were assimilated and consumed b mold. In one method of Szücs, the fungus is first grown in a m containing a sugar, phosphate, and other nutrients. It is then sepa from the growth medium by centrifuging, filtering, or decantatio introduced into the fermentation solution, which contains sugar and ent salts, with the exception of an assimilable phosphorus compoun

Martin and Waters (1952) produced citric acid from beet-s molasses media under submerged-culture conditions in tower-typ mentors (Fig. 96). The mashes were prepared by diluting the mo with water to about 12 per cent sugar, adjusting the pH to 6.0 with h chloric acid, sterilizing, adding 0.5 to 1.0 g. ferrocyanide per liter hot mash, cooling, and adding 0.1 to 1.0 g. of dibasic potassium phos trihydrate per liter. The levels of phosphate and ferrocyanide depended on the molasses samples and appeared to be interrelated. I reported that the phosphate tended to reduce the toxicity of the cyanide and thus exerted a favorable influence on citric acid produ

Steel, Lentz, and Martin (1955) found that the addition of $K_2H$ $3H_2O$ was undesirable with the samples of Chatham beet-sugar mo used by them. The addition of increasing amounts (0.01 to 0.50 g liter) of this phosphate resulted in a marked reduction in the amou

Table 152. Effect of Amount of Inoculum and Methanol Concentration on Citric Acid Production by *A. Niger* nrrl 372 in Submerged Culture *

| | 1% inoculum | | | 2% inoculum | | | 3% inoculum | | |
|---|---|---|---|---|---|---|---|---|---|
| Amount of methanol added, % | 0.1 *N* acid, ml. | Dry wt. of mycelial growth, g. | Wt. yield on glucose consumed, % | 0.1 *N* acid, ml. | Dry wt. of mycelial growth, g. | Wt. yield on glucose consumed, % | 0.1 *N* acid, ml. | Dry wt. of mycelial growth, g. | Wt. yield on glucose consumed, % |
| 1.5 | 496 | 1.22 | 37.1 | 152 | 1.12 | .... | 152 | 1.08 | |
| 2.0 | 656 | 1.15 | 47.0 | 288 | 1.15 | .... | 244 | 1.12 | |
| 2.5 | 844 | 1.01 | 63.5 | 760 | 1.12 | .... | 512 | 1.12 | |
| 3.0 | 844 | 0.93 | 69.3 | 940 | 1.04 | 69.6 | 960 | 1.05 | 69.8 |

* Courtesy of A. J. Moyer, *Appl. Microbiol.*, 1(1): 1 (1953).

Production medium, amounts per liter: glucose by analysis, 112.2 g. (11.22 g. per culture); $MgCl_2 \cdot 6H_2O$, 0.15 g.; $NaH_2PO_4 \cdot H_2O$, 0.15 g.; KCl, 0.2 g.; $NaNO_3$, 1.62 g.; $NH_4Cl$, 0.27 g.; $ZnSO_4 \cdot 7H_2O$, 0.044 g.; Fe tartrate, 0.005 g.; $MnSO_4 \cdot 4H_2O$, 0.1 g.; corn-steep liquor, 0.15 ml.

Initial pH: 4.05.

Age of inoculum: 20 hr.

Age at harvest: 9 days.

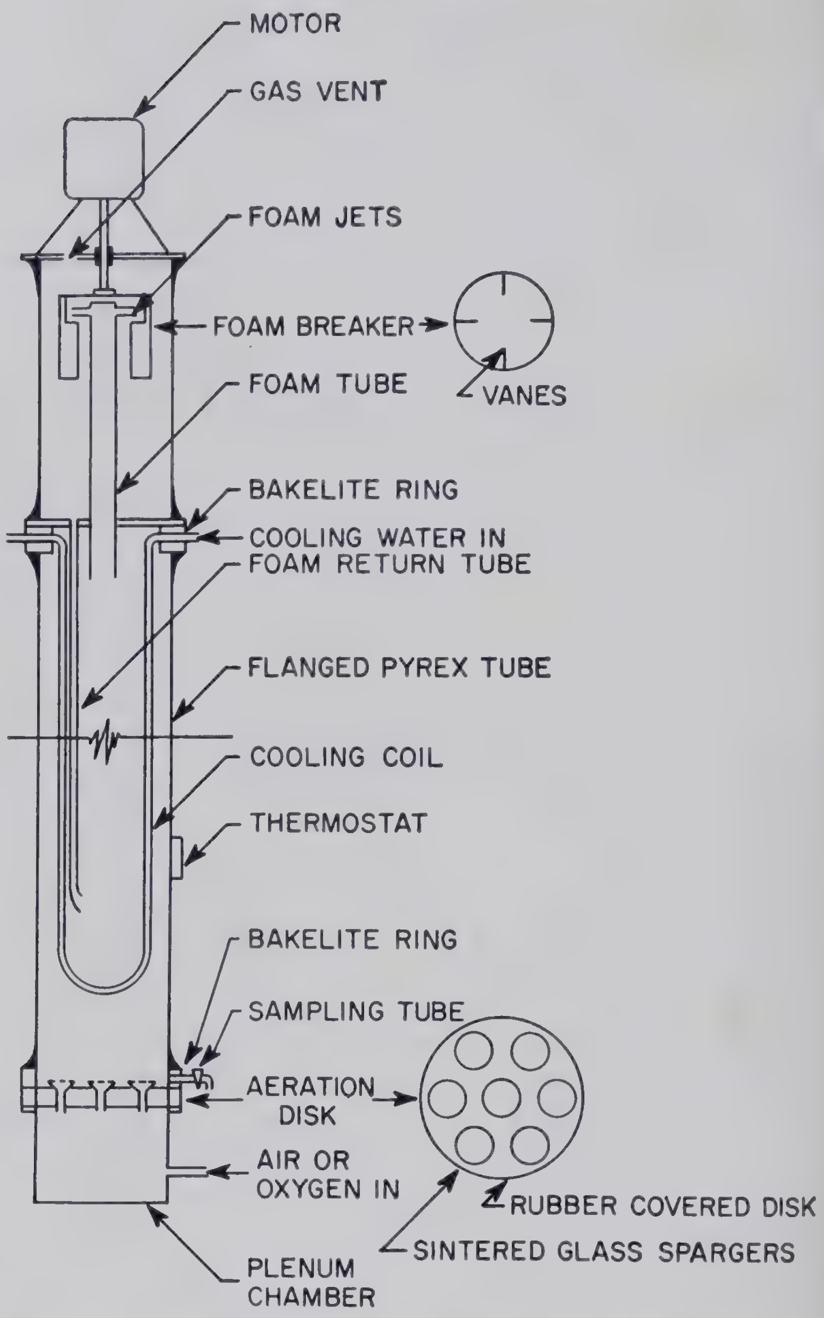

MOTOR
GAS VENT
FOAM JETS
FOAM BREAKER
VANES
FOAM TUBE
BAKELITE RING
COOLING WATER IN
FOAM RETURN TUBE
FLANGED PYREX TUBE
COOLING COIL
THERMOSTAT
BAKELITE RING
SAMPLING TUBE
AERATION DISK
AIR OR OXYGEN IN
RUBBER COVERED DISK
SINTERED GLASS SPARGERS
PLENUM CHAMBER

FIG. 96. Drawing of 36-liter fermentor. [*Courtesy of R. Steel, C. P. Lentz S. M. Martin, Can. Jour. Microbiol.,* **1**: 299 (1955).]

citric acid formed. It also resulted in the formation of large irre pellets of a mushy nature which tended to pack in the bottom o fermentor and to "cause an unstable fermentation." It was observed the phosphate affected the formation of acids other than citric acid. example, when no phosphate was added, about 80 per cent of the a other than citric, was made up of oxalic acid, but in the presence of 0 per liter of added phosphate, the amount of oxalic acid formed negligible.

Martin and Steel (1955) determined the effect of added phosphat organic acid production by *A. niger* under submerged conditions, u chromatographic analysis. Beet-sugar molasses, without added phosp with 0.5 g. of $K_2HPO_4 \cdot 3H_2O$ per liter, was fermented by the mold. ults of their findings are shown in Table 153.

will be observed that phosphate decreased the fermentation time increased the concentrations of 5-ketogluconic and gluconic acids eciably and that 5-ketogluconic was the first acid, besides citric and

153. EFFECT OF PHOSPHATE ON PRODUCTION OF ORGANIC ACIDS FROM BEET-SUGAR MOLASSES BY *A. niger* *

| | Sampling time, hr. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 10 | 24 | 30 | 45 | 58 | 72 | 116 |
| ut phosphate ermentation ne, 116 hr.): | | | | | | | | |
| ic .......... | + | + | + | ++ | ++++ | ++++ | ++++ | ++++ |
| ic .......... | + | + | + | ++ | ++ | ++ | ++ | ++ |
| togluconic .. | 0 | + | + | + | + | + | + | + |
| onic ........ | 0 | 0 | + | + | + | + | + | + |
| lic ......... | 0 | 0 | 0 | 0 | + | ++ | +++ | +++ |
| itic ........ | 0 | 0 | 0 | 0 | 0 | + | + | + |
| aric ........ | 0 | 0 | 0 | 0 | 0 | + | + | + |
| iown ($X_2$) .. | 0 | 0 | 0 | 0 | 0 | + | + | + |
| hosphate rmentation ie, 72 hr.): | | | | | | | | |
| .......... | + | + | ++ | ++ | ++++ | ++++ | ++++ | |
| .......... | + | + | + | ++ | ++ | ++ | ++ | |
| ogluconic .. | 0 | ++ | ++ | ++ | ++ | +++ | +++ | |
| nic ........ | 0 | 0 | ++ | ++ | ++ | +++ | +++ | |
| .......... | 0 | 0 | 0 | 0 | 0 | 0 | + | |
| tic ........ | 0 | 0 | 0 | 0 | + | + | + | |
| ric ........ | 0 | 0 | 0 | 0 | + | + | + | |
| own ($X_2$)... | 0 | 0 | 0 | 0 | + | + | + | |

. Martin and R. Steel, *Can. Jour. Microbiol.*, 1: 470–472 (1955).

0 Undetected.
+ Trace (< 0.1 per cent).
+ Low (about 0.2 per cent).
+ Medium (about 0.5–1.5 per cent).
+ High (< 3.0 per cent).

icids, to appear in each fermentation. It will be noted also that icid accumulated in the medium without added phosphate after he forty-fifth hour and was present in relatively high concentra- the end of the fermentation. However, in the presence of added ite, only trace amounts of this acid were found at the end of the ation.

**iration of Inoculum.** Procedures for preparing inocula have been d by Perquin (1938), Szücs (1944), Karow and Waksman (1947), (1953), Steel et al. (1954), and others.

*Method of Moyer.* In the procedure developed by Moyer, t cultures are maintained on Czapek's agar slants, while sporula germination of the spores are induced in special media (refer t 154).

TABLE 154. MEDIA USED IN PREPARATION OF INOCULUM

| | Sporulation medium | Germinatio medium |
|---|---|---|
| Commercial glucose | 30.0 g. | 50.0 g. |
| Beet molasses | 20.0 g. | |
| Urea | 0.3 g. | |
| $NH_4NO_3$ | ........ | 1.0 g. |
| $(NH_4)_2HPO_4$ | 0.6 g. | |
| $KH_2PO_4$ | 0.15 g. | 0.25 g. |
| KCl | 0.2 g. | |
| $MgSO_4 \cdot 7H_2O$ | 0.13 g. | 0.1 g. |
| Fe tartrate | 0.005 g. | 0.005 g. |
| $MnSO_4 \cdot 4H_2O$ | 0.02 g. | |
| $CuSO_4 \cdot 5H_2O$ | 0.005 g. | |
| Corn-steep liquor | 3.0 g. | 0.8 ml. |
| $ZnSO_4 \cdot 7H_2O$ | ........ | 0.44 g. |
| $CaCO_3$ | 0.5 g. | |
| Agar | 40.0 g. | * |
| Tap water | 200 ml. | |
| Distilled water to make | 1,000 ml. | 900 ml. |

* 0.4 g. agar is added to each 300-ml. Erlenmeyer flask containing 90 ml. of m before sterilization. Each flask is agitated immediately after removal fro autoclave to ensure even distribution of the agar.

Slants of the sporulation medium are inoculated with *A. niger* and bated at 32°C. for 7 days. Then 10 g. of sterile, dry, sharp, white sa poured over the surface growth, which should occupy about 15 sq The growth is stirred with the sand and with a sterile needle to d and separate the spores. Next 25 ml. of sterile soap solution (0.1 Ivory soap in 300 ml. of distilled water) is used to wash off the sp The spore suspension thus obtained is used to inoculate two 30 Erlenmeyer flasks, each of which contains 90 ml. of sterile germin medium. The flasks are incubated for about 22 hr. at 30 to 32°C. being agitated on a shaker. By the end of this time, each spore us has produced two germ tubes. Pellet formation is undesirable. Ordin 1 to 2 per cent by volume of germinated spore inoculum is used for purposes.

*Method of Steel, Martin, and Lentz.* A method for preparing a st ard inoculum has been developed by Steel and associates (1954). A n tionally rich sporulation medium of the following composition was pared, sterilized, distributed in plates, inoculated, and incubated un good crop of spores resulted:

| | |
|---|---|
| Molasses | 300 g. |
| $KH_2PO_4$ | 0.5 g. |
| Agar | 20 g. |
| Distilled water to | 1,000 ml. |
| Poststerilization pH | 6 |

spore crop on a plate was suspended in 10 ml. of a sterile 0.05 per aqueous solution of Aerosol O.T. The spore suspension obtained was ed into a sterile container. The plate was then washed twice with e water, and the washings were combined with the main part of uspension. The volume of the suspension was made up to 100 The suspension was agitated with glass beads to facilitate the sepa- n of the individual spores. It usually contained 45 to 55 × $10^6$ spores l.

e general procedure for preparing the seed cultures was as follows: sh was made from uniformly mixed beet-sugar molasses diluted to a ntration of about 13 per cent with tap water and adjusted to pH 6.0 ydrochloric acid. The desired amount of $K_4Fe(CN)_6 \cdot 3H_2O$ (as an us solution) was added to 1,500 ml. of the mash, which was auto- l at 15 p.s.i.g. for 25 min. and then cooled to room temperature. cooling, the pH of the mash was adjusted to the desired level, and $_2HPO_4 \cdot 3H_2O$ (in the form of a sterile solution) was added to yield entration of 0.5 g. per liter. This medium was dispensed aseptically -ml. amounts into sterile 1-liter flasks, inoculated with a specified r of spores, and incubated at 27°C. on a Gump rotary shaker (at .m.).

development of a standard type of pellet (refer to Fig. 97) was . The standard pellet varied from 0.2 to 0.5 mm. in diameter. 155 to 157 show the relationship of ferrocyanide and pH, ferro-

ABLE 155. EFFECT OF FERROCYANIDE AND pH ON PELLET DEVELOPMENT *

(600 × $10^6$ spores per culture)

| N)6·$3H_2O$, /liter | pH | | | |
|---|---|---|---|---|
| | 5.0 | 6.0 | 7.0 | 8.0 |
| ).30 | Retarded | Filamentous | Filamentous | Retarded |
| 0.35 | Retarded | Poststandard | Standard | Retarded |
| 0.40 | Retarded | *Standard* | Prestandard | Retarded |
| ).45 | Retarded | Prestandard | Prestandard | Retarded |

eel, S. M. Martin, and C. P. Lentz, *Can. Jour. Microbiol.*, 1: 150 (1954).

and numbers of spores, and pH and numbers of spores, respec- n pellet development. It will be noted that the effects of ferrocya- I, and numbers of spores are related and that, to a certain extent, e in one level may be compensated for by a change in another. le combinations of these factors for the production of a standard

TABLE 156. EFFECT OF FERROCYANIDE AND SPORE LEVEL ON PELLET DEVEL

(pH 6.0)

| $K_4Fe(CN)6 \cdot 3H_2O$, g./liter | Spores per culture × $10^6$ | | | |
|---|---|---|---|---|
| | 450 | 600 | 750 | |
| 0.30 | Filamentous | Filamentous | Filamentous | Fil |
| 0.35 | Prestandard | Standard | Standard | Po |
| 0.40 | Prestandard | *Standard* | Standard | Po |
| 0.45 | Prestandard | Prestandard | Prestandard | Po |

* R. Steel, S. M. Martin, and C. P. Lentz, *Can. Jour. Microbiol.*, **1**: 150 (

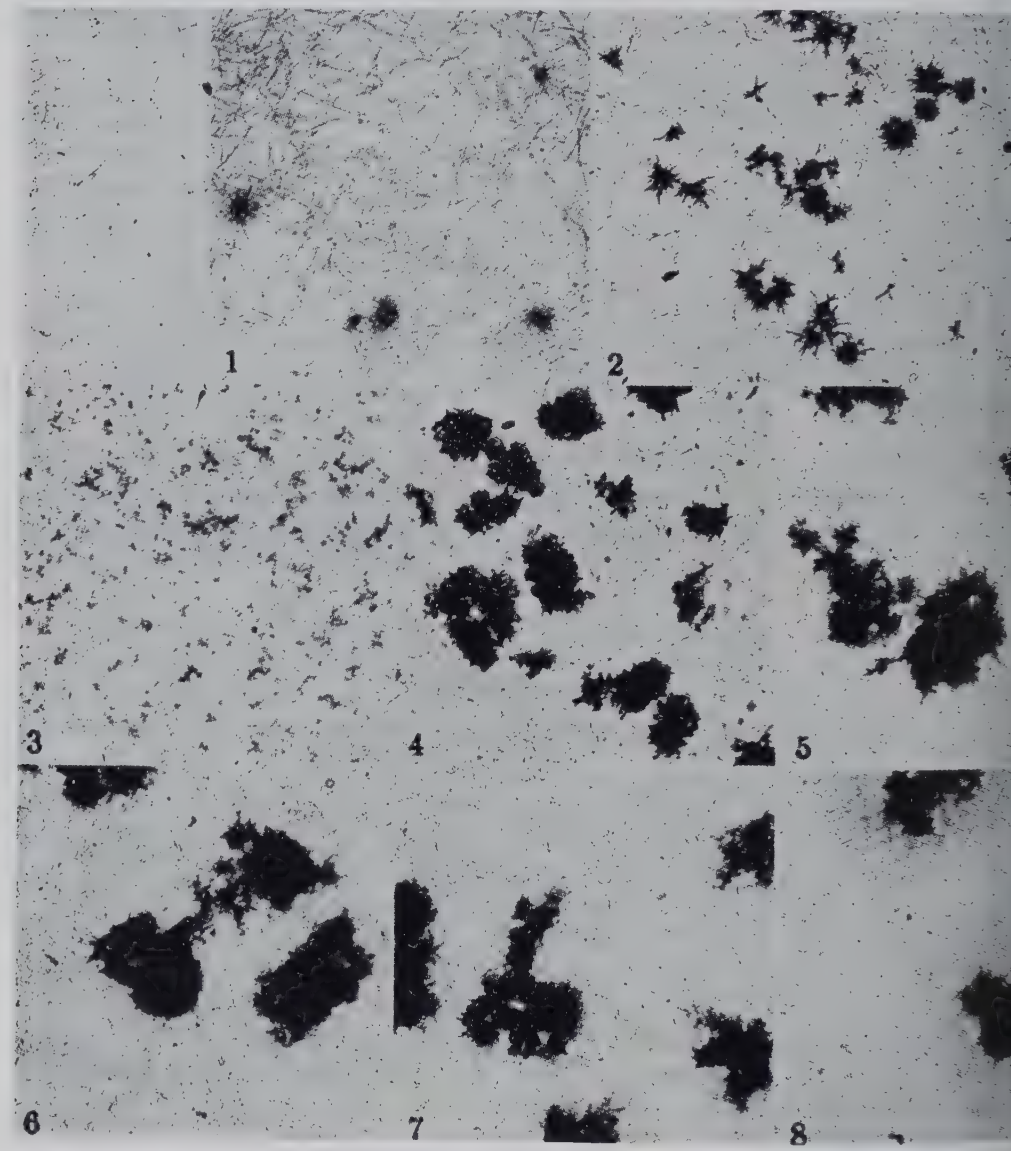


FIG. 97. Appearances of inoculum at different stages of development: 1, filar
type; 2, retarded type; 3–8, developmental stages of standard pellet type; 3.
ture stage (14 hr.); 4, early prestandard stage (16 hr.); 5. middle prestandar
(18 hr.); 6, late prestandard stage (20 hr.); 7, standard stage (22 hr.); and
standard stage (25 hr.). [*Courtesy of R. Steel, S. M. Martin, and C. P. Lent*
*Jour. Microbiol.*, **1**: 150 (1954).]

TABLE 157. EFFECT OF pH AND SPORES ON PELLET DEVELOPMENT *
(0.4 g. $K_4Fe(CN)_6 \cdot 3H_2O$ per liter)

| pH | Spores per culture × $10^6$ | | | |
|---|---|---|---|---|
| | 450 | 600 | 750 | 900 |
| 5.0 | Filamentous | Filamentous | Filamentous | Filamentous |
| 6.0 | Prestandard | *Standard* | Standard | Poststandard |
| 7.0 | Retarded | Prestandard | Prestandard | Standard |
| 8.0 | Retarded | Retarded | Retarded | Retarded |

Steel, S. M. Martin, and C. P. Lentz, *Can. Jour. Microbiol.*, 1: 150 (1954).

lum are noted in these tables. Steel and coworkers found that differevels were optimum for molasses of different crop years and for ent lots within a given crop year.

**ect of Treatment of Commercial Sugars with Ion-exchange ts.** Perlman, Dorrell, and Johnson (1946) found that commercial s of sucrose and glucose gave low yields of citric acid when ferd by *A. niger* 62, owing to the metallic ions contained. However, he sugars had been treated by passage over cationic-exchange mateuch as "Alkalex" (Research Products Corp., Madison, Wis.), "Am- IR-100" (Resinous Products & Chemicals Co., Philadelphia), Zeo-Karb H" (Permutit Co., New York), the yields were increased han threefold (refer to Table 158).

E 158. EFFECT OF TREATMENT OF COMMERCIAL SUGARS BY IONIC-EXCHANGE METHOD ON ACID FORMATION (STRAIN 62)*

| gar | Treatment | Yield of citric acid, per cent | Yield of oxalic acid, per cent | Sugar fermented, per cent | Length of fermentation, days |
|---|---|---|---|---|---|
| crose ... | None | 21.4 † | ... | 34.4 | 8 |
| | Cationic exchange | 64.0 | 4.1 | 98.0 | 8 |
| rose..... | None | 11.3 † | ... | 17.6 | 8 |
| | Cationic exchange | 66.8 | 5.6 | 97.5 | 10 |
| ‡ ....... | None | 20.5 † | ... | 33.2 | 8 |
| | Cationic exchange | 65.0 | 7.2 | 99.0 | 10 |

rlman, W. W. Dorrell, and M. J. Johnson, *Arch. Biochem.*, 10(3): 131 (1946).
icated in medium A were used. Yields reported are for day of maximum citric acid.
table acidity calculated as citric acid.
lose" (glucose monohydrate).

**action Data.** Some data concerning the production of citric acid ubmerged process are presented in Table 159.

Table 159. Some Data concerning the Production of Citric Acid by the Submerged Process

| Carbohydrate source | Reagent used in preparation of substrate | Yield, % | Duration of fermentation | Reference |
|---|---|---|---|---|
| Beet molasses | Potassium ferrocyanide | 64 (max.)* | 8–9 days | Clement, M. T.: *Can. Jour. Technol.*, **30**: 82 (1952). |
| | Potassium ferrocyanide | 72 (max.)* | 70 hr. | Martin, S. M., and W. R. Waters: *Ind. Eng. Chem.*, **44**: 2229 (1952). |
| | Potassium ferrocyanide | 68.5 * | 116 hr. | Steel, R., S. M. Martin, and C. P. Lentz: *Can. Jour. Microbiol.*, **1**: 150 (1954). |
| | Potassium ferrocyanide | 64 (avg.)* | 80–116 hr. | ———, C. P. Lentz, and S. M. Martin: *Can. Jour. Microbiol.*, **1**: 299 (1955). |
| Cane molasses | Methanol | 63.3 † | 9 days | Moyer, A. J.: *Appl. Microbiol.*, **1**: 7 (1953). |
| | Bone char + Suchar CSP + Amberlites | 61.9 † | 12 days | Karow, E. O., and S. A. Waksman: *Ind. Eng. Chem.*, **39**: 821 (1947). |
| Cornstarch | Methanol | 71.5 † | 10 days | Moyer, A. J.: *Appl. Microbiol.*, **1**: 7 (1953). |
| Corn sirup | Morpholine | 79.9 | 8.67 days | Schweiger, L. B., and R. L. Snell: U.S. Patent 2,476,159, July 12, 1949. |
| Glucose | Methanol | 71.2 † | 7 days | Moyer, A. J.: *Appl. Microbiol.*, **1**: 7 (1953). |
| Sucrose | | 72 (avg.)* | 9 days | Shu, P., and M. J. Johnson: *Ind. Eng. Chem.*, **40**: 1202 (1948). |

* Based on available sugar.
† Based on sugar consumed.

## ORIES CONCERNING THE MECHANISM OF CITRIC ACID FORMATION

though many theories have been advanced to explain the formation ric acid from carbon-containing compounds, none proposed has satis- rily explained all the known observations. Various theories are here nted to show the complexity of the reactions that may occur in this ntation rather than to assume that any one of them shows the true c. Any theory proposed must account for the production of citric from 2-, 3-, 4-, 5-, 6-, 7-, and 12-carbon compounds if it is to be table. It must account for high yields of citric acid from sugars, in some instances have approached 100 per cent on the basis of igar consumed.

ze and Perrier (1904) suggested that citric acid arose as a product omplete respiratory metabolism.

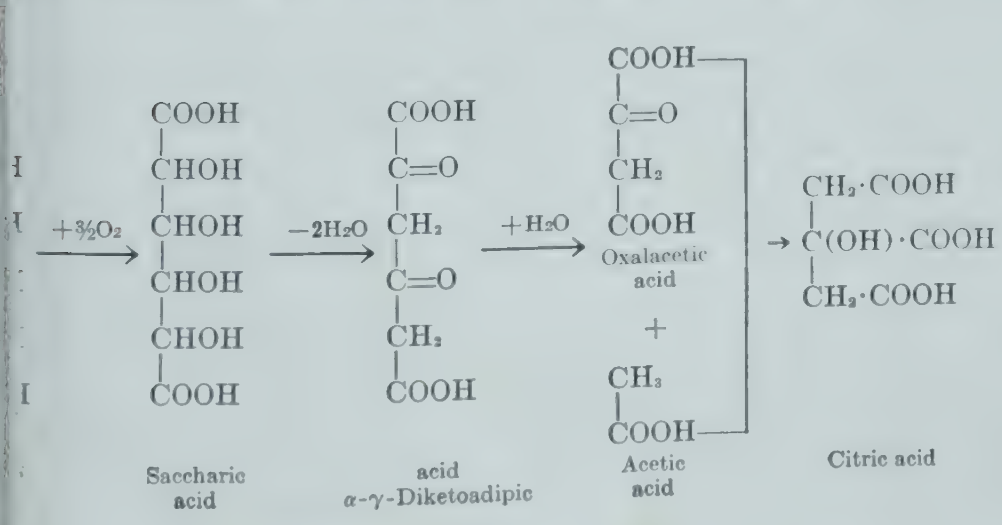
asaccharinic acid played an important part as an intermediate in ry proposed by Buchner and Wustenfeld. Sugar was broken down asaccharinic acid, which was rearranged to form citric acid. A con- tion reaction would be necessary to explain citric acid formation some sugars (Herzog and Polotsky, 1909).

er (1909) and others suggested that pyruvic acid was formed from and that this was decomposed to acetaldehyde and carbon dioxide. molecules of acetaldehyde condensed and the product was oxidized ic acid:

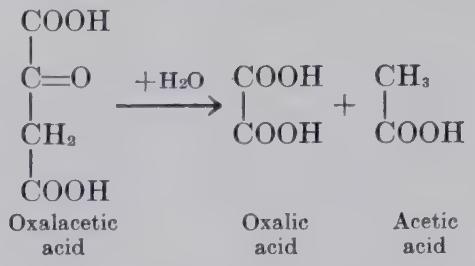
$$\underset{\text{Glucose}}{3C_6H_{12}O_6} \rightarrow \underset{\text{Pyruvic acid}}{6CH_3CO\cdot COOH} \rightarrow 6CO_2 + \underset{\text{Citric acid}}{2C_6H_8O_7}$$

maximum yield by this theory could only be 71.1 per cent of citric herefore this hypothesis can be eliminated on quantitative grounds

919, Raistrick and Clark suggested that hexose is broken down a-gamma-diketoadipic acid, which then becomes hydrolyzed to and oxalacetic acid, these two acids combining to form citric

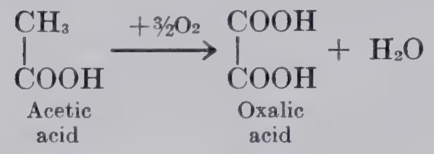


Oxalic acid may arise from the breakdown of oxalacetic acid oxidation of acetic acid:



$$\begin{array}{c}\mathrm{COOH}\\|\\\mathrm{C{=}O}\\|\\\mathrm{CH_2}\\|\\\mathrm{COOH}\end{array} \xrightarrow{+H_2O} \begin{array}{c}\mathrm{COOH}\\|\\\mathrm{COOH}\end{array} + \begin{array}{c}\mathrm{CH_3}\\|\\\mathrm{COOH}\end{array}$$

Oxalacetic acid — Oxalic acid — Acetic acid



$$\begin{array}{c}\mathrm{CH_3}\\|\\\mathrm{COOH}\end{array} \xrightarrow{+\frac{3}{2}O_2} \begin{array}{c}\mathrm{COOH}\\|\\\mathrm{COOH}\end{array} + H_2O$$

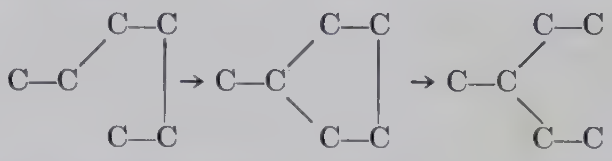
Acetic acid — Oxalic acid

Although this theory can account for the high yields of citric ac a hexose, it does not account for the production of this acid fro stances other than a hexose, unless it is assumed that a hexose is sized in each case.

In 1924, Butkewitsch proposed a scheme in which glucose was oxidized to citric acid:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} + \tfrac{3}{2}O_2 \rightarrow \underset{\text{Citric acid}}{C_6H_8O_7} + 2H_2O$$

Butkewitsch was of the opinion that glucose passed first to gluconi which was subsequently transformed to glucuronic acid. The latt then supposed to undergo an intramolecular aldol condensation to five-membered ring. Rupture of the ring, followed by oxidation, led formation of citric acid:



Wehmer (1925) suggested the possibility of the following rea taking place, since calcium gluconate was fermented by *Aspergillus* to citric acid:

Glucose → Gluconic acid → Citric acid → Oxalic acid → Carbon dioxide

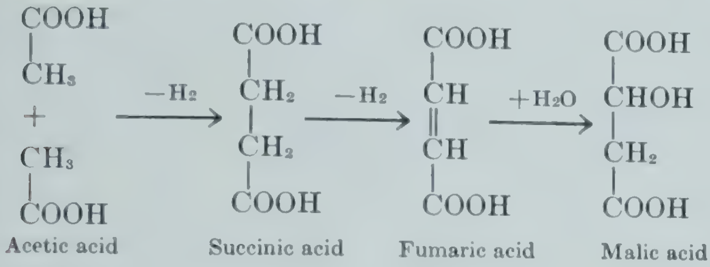
Saccharic acid was the principal intermediate product in a theor gested by Franzen and Schmitt (1925):

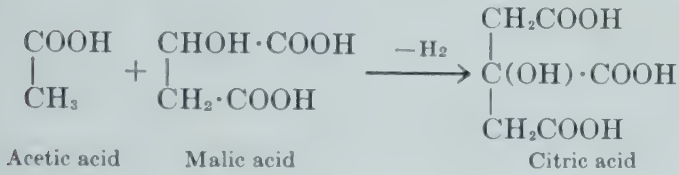
$$\text{Sugar} \rightarrow \text{Saccharic acid} \rightarrow \beta\text{-}\gamma\text{-Diketoadipic acid} \xrightarrow[\text{acid rearrangement}]{\text{by benzilic}} \text{Citric}$$

Challenger and his associates (1927) added weight to this theor they isolated saccharic acid from media containing glucose that had fermented by *A. niger*. Calcium gluconate also yielded sacchari citric acids. Citric acid was obtained from potassium hydrogen sa rate solutions inoculated with *A. niger*. Bernhauer, however, not believe that saccharic acid is an intermediate product in the

fermentation, as the result of his work with a large number of
s.

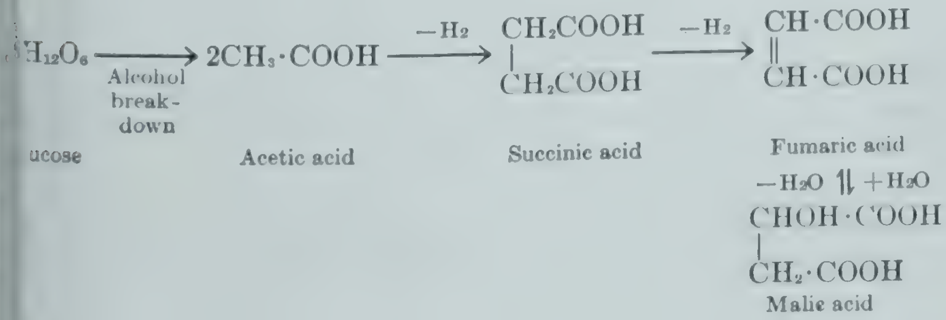
**eory of Chrzaszcz and Tiukow.** From the observations (1930)
certain molds have the ability to produce citric acid, along with
nic, fumaric, and malic acids, from acetic acid and ethyl alcohol,
aszcz and Tiukow have formulated their scheme for the formation
ese acids. Two molecules of acetic acid are dehydrogenated to 1
ule of succinic acid, which, in turn, by loss of hydrogen, passes to
ric acid. This acid is converted to malic acid upon the addition of 1
ule of water. One molecule of malic acid and 1 molecule of acetic
ombine with the loss of hydrogen to form citric acid.

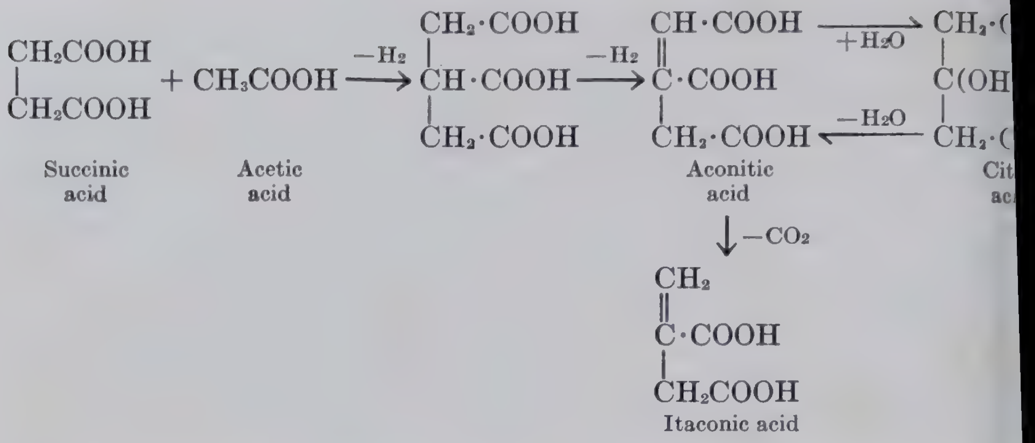

COOH
CH3
+
CH3
COOH
−H2
COOH
CH2
CH2
COOH
−H2
COOH
CH
CH
COOH
+H2O
COOH
CHOH
CH2
COOH
Acetic acid
Succinic acid
Fumaric acid
Malic acid


COOH
CH3
+
CHOH·COOH
CH2·COOH
−H2
CH2COOH
C(OH)·COOH
CH2COOH
Acetic acid
Malic acid
Citric acid

as later suggested that the initial stage in the production of citric
om sugars was similar to that of ethyl alcohol fermentation. If
t were true, then the maximum yield of citric acid from glucose
not exceed 71.1 per cent and the ratio of the weight of citric acid to
dioxide should not exceed 1.45:1. Wells and his associates have
definitely by the use of carbon-balance experiments that yields
rably higher than 71.1 per cent may be obtained from glucose and
t the ratio 1.45:1 is greatly exceeded. These general observations
en corroborated by others.

**hauer's Theory.** Bernhauer, Böckl, and Siebenauger (1932) have
d that acetic acid and ethyl alcohol are formed from sugars by
n the manner indicated by Neuberg's scheme for alcohol produc-
yeasts. Acetic acid is converted to citric acid through aconitic
follows:

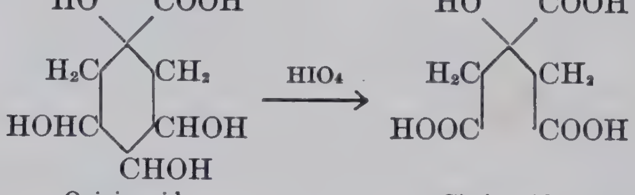

H12O6
Alcohol break-down
2CH3·COOH
−H2
CH2COOH
CH2COOH
−H2
CH·COOH
CH·COOH
ucose
Acetic acid
Succinic acid
Fumaric acid
−H2O
+H2O
CHOH·COOH
CH2·COOH
Malic acid



The preceding scheme is based on several facts (see the th Chrzaszcz and Tiukow). Aconitic acid may be converted to citr by molds producing citric acid (one strain having formed over cent citric acid from aconitic acid) while citric acid may be trans to aconitic acid. Methylglyoxal has been produced from sodium diphosphate by aspergilli, which have the ability to form citric acid

This theory is not acceptable, however, because the maximum of citric acid by this scheme cannot exceed 71.1 per cent and the r the weights of citric acid and carbon dioxide cannot exceed Furthermore aconitic acid has not been isolated from fermentati sugars by molds, although *A. itaconicus* produces itaconic, citric gluconic acids from sucrose and fructose.

**Theory of Emde.** Emde's scheme (1935) for the conversion of s to citric acid through quinic acid was proposed after it had been sho Fischer and Dangschat that quinic acid could be oxidized to citric by periodic acid.



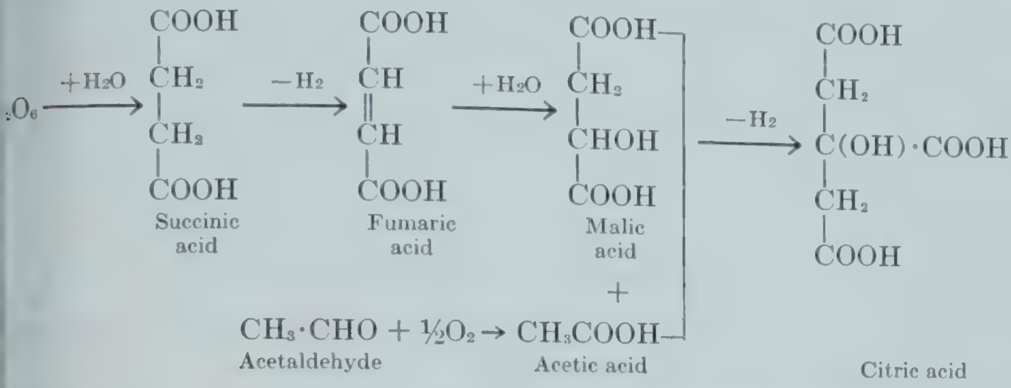
The maximum possible yield of citric acid from sucrose, accordi Emde's scheme, is 56.1 per cent:

$$\underset{\text{Sucrose}}{C_{12}H_{22}O_{11}} + 5O_2 \rightarrow \underset{\text{Quinic acid}}{C_7H_{12}O_6} + 5CO_2 + 5H_2O$$

$$\underset{\text{Quinic acid}}{C_7H_{12}O_6} + 5/2O_2 \rightarrow \underset{\text{Citric acid}}{C_6H_8O_7} + CO_2 + 2H_2O$$

Therefore, on the basis of yields alone, this theory is untenable. B witsch, who had suggested that only molds with the ability to pro citric acid were able to ferment quinic acid, was unable to obtain acid from quinic acid by fermentation.

**Theory of Gudlet.** The scheme proposed by Gudlet (1935) is b on the fact that glucose may split directly into a 4-carbon mole nic acid, and a 2-carbon compound, acetaldehyde. (Virtanen origi- suggested that this reaction might take place in the propionic acid ntation.)



high yields obtained from glucose, on the basis of the sugar con- l, could be explained by this scheme since decarboxylation is not ned.

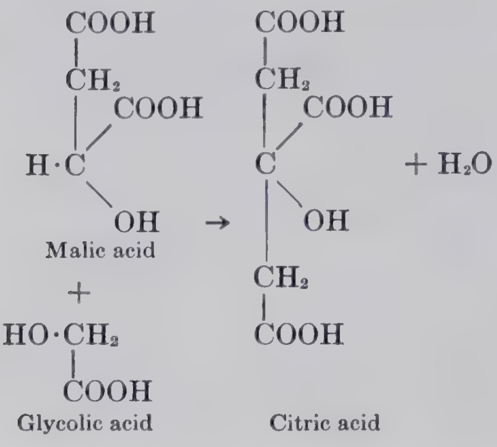
theory that acetic acid is directly converted to succinic acid and acid has been attacked by Butkewitsch, Menzshinskaya, and Trofi- (1934), who hold that citric acid is formed from substances of the ium by *A. niger*, the process being stimulated by the addition of acid. These investigators state that there is no direct connection n the consumption of acetic acid and the accumulation of citric xalic acid being the principal substance produced from the conver- acetic acid.

carefully weighing the mycelium, citric acid, acetic acid, and other ts involved, Bernhauer and others have secured information by hey have been able to refute the hypothesis of citric acid formation ubstances of the mycelium.

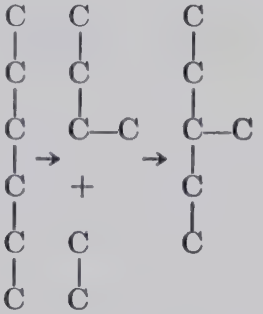
her hypothesis concerning the fermentation of acetic acid is that ed in the synthesis of carbohydrates which are later broken down c or succinic acids. This theory has little evidence for support.

n citric acid is fermented anaerobically with yeast, each molecule c acid yields 2 molecules of acetic acid, 2 molecules of carbon , and some formic acid. *Salmonella aertrycke* decomposes citric to acetic, succinic, and formic acids. These facts indicate a pos- nnection of citric acid with acetic, succinic, and other acids.

**ory of Ciusa and Brüll.** Ciusa and Brüll have shown that the n of malic acid, glycolic acid, or mixtures of the two acids to t sugar solutions, adjusted to a pH of 3.5, increased the yields of cid produced from sugar by *A. niger*. The addition of malic acid d the yield to 332 per cent of the sugar consumed, while glycolic reased the yield to 132 per cent. When equimolar quantities of nd glycolic acid were added, the yield was increased to 928 per n the basis of these results, Ciusa and Brüll suggested that the se of the intermediary reactions in the citric acid fermentation e a condensation of malic and glycolic acids:



The citric acid fermentation might be schematically repres
follows, according to Ciusa and Brüll:



## THE KREBS CYCLE AND CITRIC ACID FORMATION

Recent research has supplied information on the relationship
Krebs citric acid cycle to the formation of citric acid by *Aspergillu*
Lewis and Weinhouse (1951), as a result of using labeled aceta

TABLE 160. ENZYMES OF THE TRICARBOXYLIC ACID CYCLE WHICH HAVE B
DEMONSTRATED IN *Aspergillus niger*

| Enzyme | Reference |
|---|---|
| Succinoxidase system | Martin, S. M.: *Can. Jour. Microbiol.,* (1954). |
| Condensing enzyme | Ramakrishnan, C. V., and S. M. Martin: *Jour. Biochem. Physiol.,* **32**: 434 (1954). |
| Isocitric dehydrogenase | Ramakrishnan, C. V., and S. M. Martin: *Biochem. and Biophys.,* **55**: 403 (1955). |
| Aconitase<br>α-Ketoglutaric oxidase<br>Fumarase<br>Malic dehydrogenase<br>Malic enzyme<br>Pyruvic oxidase<br>Acetate activating enzyme<br>Transacetylase | Ramakrishnan, C. V.: *Enzymologia,* 17 3): 169 (1954). |

ium, suggested that citric acid was formed as an "overflow product" ng the faulty operation of the Krebs cycle. Martin (1954), Rama- man and Martin (1955), and Ramakrishnan (1954) showed that all nzymes of the Krebs citric acid cycle could be demonstrated in cell- extracts of *A. niger* when grown in a non-citrate-accumulating um (refer to Table 160). Ramakrishnan, Steel, and Lentz (1955) ined cell-free extracts of the pellets of *A. niger* which were grown citrate-accumulating medium.

simplified diagram of the Krebs cycle is shown in Fig. 98. A more led demonstration and explanation of the cycle may be found in *ht Air Development Center Technical Report* 52-301.

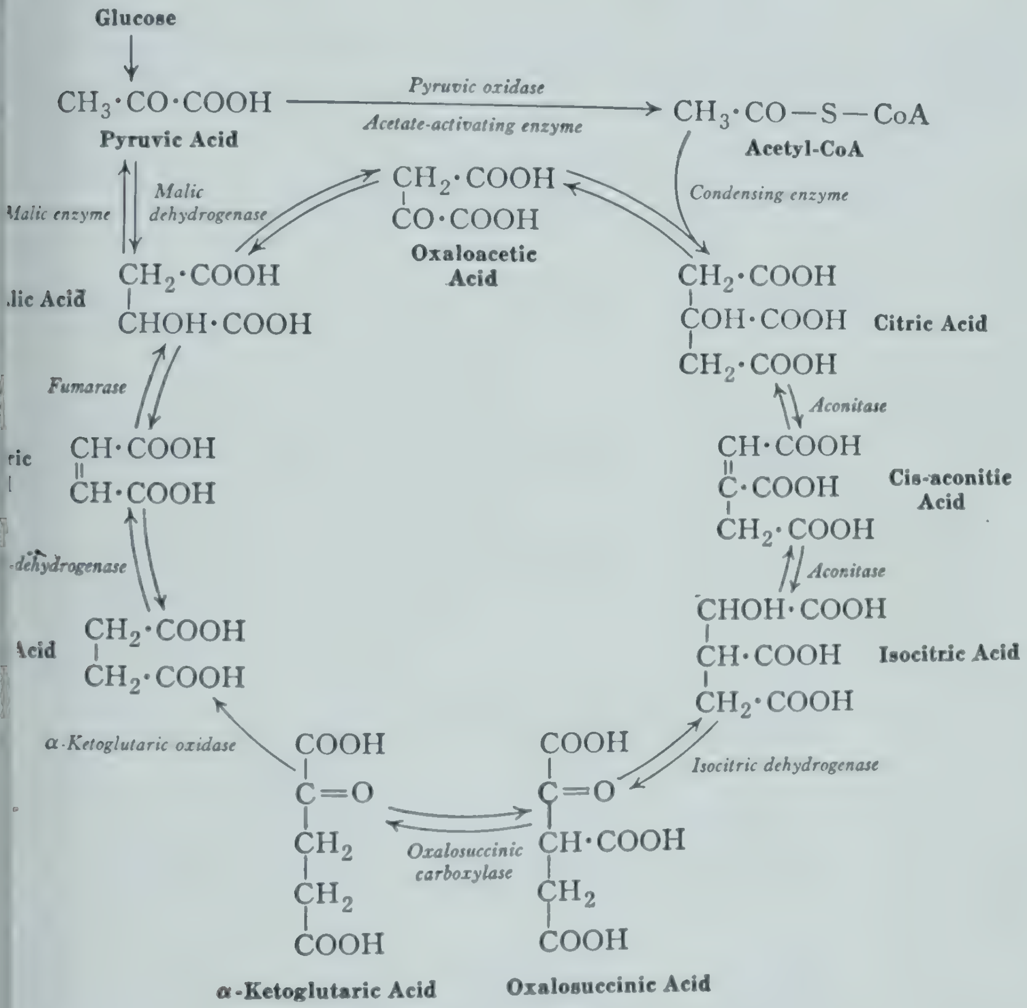

Glucose
CH3·CO·COOH
Pyruvic Acid
Pyruvic oxidase
Acetate-activating enzyme
CH3·CO—S—CoA
Acetyl-CoA
Condensing enzyme
Malic enzyme
Malic dehydrogenase
CH2·COOH
CO·COOH
Oxaloacetic Acid
CH2·COOH
CHOH·COOH
lic Acid
CH2·COOH
COH·COOH
CH2·COOH
Citric Acid
Aconitase
Fumarase
CH·COOH
CH·COOH
ric
CH·COOH
C·COOH
CH2·COOH
Cis-aconitic Acid
-dehydrogenase
Aconitase
CH2·COOH
CH2·COOH
Acid
CHOH·COOH
CH·COOH
CH2·COOH
Isocitric Acid
α-Ketoglutaric oxidase
COOH
C=O
CH2
CH2
COOH
Oxalosuccinic carboxylase
COOH
C=O
CH·COOH
CH2
COOH
Isocitric dehydrogenase
α-Ketoglutaric Acid
Oxalosuccinic Acid

FIG. 98. Krebs cycle.

· 161 shows the effect of fermentation time, potassium ferrocya- nd phosphate on the specific activities of enzymes of *A. niger* re known to exercise a role in the tricarboxylic acid cycle. Rama- n et al. (1955) grew the mold pellets in citric-acid-accumulating nd at specified intervals (refer to Table 161) withdrew samples. lets were washed in glass-distilled water until they were free of and then squeezed in cheesecloth to remove as much of the water le. Cell-free extracts of the pellets were employed to determine

Table 161. Effect of Fermentation Time, Potassium Ferrocyanide, and Phosphate on the Specific Activities of Oxidizing Enzymes of *A. niger* *

| Conditions | Specific activity, units/mg. protein | | | | | | | | | | Citric acid yield, g./100 ml. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Acetate-activating enzyme | Trans-acetylase | Condensing enzyme | Aconitase | Isocitric dehydrogenase | α-Keto-glutaric oxidase | Fumarase | Malic dehydrogenase | Pyruvic oxidase | Malic enzyme | |
| Fermentation time, hr.: | | | | | | | | | | | |
| 24 | 0.03 | 0.10 | 0.62 | 18.2 | 12.7 | 0.5 | 10.1 | 15.2 | 0.1 | 10.2 | 0.0 |
| 46 | 0.07 | 5.0 | 2.0 | 28.0 | 0.0 | 1.2 | 20.0 | 40.0 | 0.8 | 0.0 | 3.1 |
| 93 | 0.10 | 4.4 | 6.0 | 0.0 | 0.0 | 6.0 | 42.0 | 43.5 | 4.2 | 0.0 | 6.5 |
| 120 | 0.05 | 2.0 | 5.7 | 0.0 | 0.0 | 4.1 | 35.0 | 22.0 | 1.9 | 0.0 | 7.1 |
| Ferrocyanide, g./liter: † | | | | | | | | | | | |
| 0.0 | 0.02 | 0.20 | 1.04 | 0.0 | 0.0 | 0.0 | 23.0 | 0.0 | 0.0 | 0.0 | 0.5 |
| 0.3 | 0.38 | 5.00 | 16.0 | 0.0 | 0.0 | 4.2 | 60.0 | 15.8 | 1.2 | 0.0 | 5.4 |
| 0.5 | 0.10 | 4.2 | 6.0 | 0.0 | 0.0 | 5.8 | 42.0 | 43.0 | 4.2 | 0.0 | 5.4 |
| Phosphate, g./liter: ‡ | | | | | | | | | | | |
| 0.0 | 0.08 | 3.0 | 5.8 | 0.0 | 0.0 | 5.2 | 38.0 | 29.0 | 3.8 | 0.0 | 9.6 |
| 0.01 | 0.05 | 1.8 | 11.4 | 0.0 | 0.0 | 5.4 | 48.0 | 20.0 | 4.8 | 0.0 | 9.0 |
| 0.25 | 0.09 | 1.5 | 0.0 | 37.0 | 1.8 | 0.0 | 30.0 | 0.0 | 0.0 | 0.0 | 0.8 |
| 0.50 | 0.05 | 1.5 | 0.0 | 107.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

activities of the enzymes present, using the procedures described by nakrishnan (1954).

n examination of the table shows that the enzymes of the Krebs citric l cycle were present in the cell-free extracts obtained from the mold ets grown for 24 hr. in the fermentation medium. It is to be noted no citric acid accumulated during the first 24 hr. At the end of ır., when citric acid had commenced to accumulate, it will be observed there was an increase in the activity of the condensing enzyme and a ppearance of the activity of the isocitric dehydrogenase. After 93 hr. during the period of maximum citric accumulation, the activity of condensing enzyme had increased by almost 10 times, and no activi- for isocitric dehydrogenase or aconitase were observed.

ie beneficial effects of ferrocyanide and low concentrations of added phate may also be observed by reference to Table 161. These data ate that the specific activity of the condensing enzyme increased the activities of the isocitric dehydrogenase and aconitase disap- ed during the period of citric acid formation, which strongly sug- an interruption of the Krebs cycle at the level of the latter two mes.

me of the reactions believed to occur in the Krebs cycle are shown v.

$$\text{Pyruvate} \xrightarrow{\text{Pyruvic oxidase}} \text{Acetate} + CO_2$$

$$\text{Acetate} + \text{ATP} + \text{CoA} \xrightarrow{\text{Acetate activating enzyme}} \text{Acetyl-CoA} + \text{AMP}$$

$$\text{Acetyl phosphate} + \text{CoA} \overset{\text{Transacetylase}}{\rightleftarrows} \text{Acetyl-CoA} + \text{Phosphate}$$

$$\text{Pyruvate} + CO_2 \overset{\text{Oxalacetic decarboxylase}}{\rightleftarrows} \text{Oxalacetate}$$

$$\text{Acetyl-CoA} + \text{Oxalacetate} \overset{\text{Condensing enzyme}}{\rightleftarrows} \text{Citrate}$$

$$\text{Citrate} \underset{(+H_2O)}{\overset{\text{Aconitase } (-H_2O)}{\rightleftarrows}} \text{Cis-aconitate} \underset{(-H_2O)}{\overset{\text{Aconitase } (+H_2O)}{\rightleftarrows}} \text{Isocitrate}$$

$$\text{Isocitrate} \underset{(+H_2)}{\overset{\text{Isocitric dehydrogenase } (-H_2)}{\rightleftarrows}} \text{Oxalosuccinate}$$

$$\text{Oxalosuccinate} \underset{(+CO_2)}{\overset{\text{Oxalosuccinic decarboxylase } (-CO_2)}{\rightleftarrows}} \alpha\text{-Ketoglutarate}$$

$$\alpha\text{-Ketoglutarate} \xrightarrow{\alpha\text{-Ketoglutaric oxidase } (+\frac{1}{2}O_2)} \text{Succinate} + CO_2$$

$$\text{Succinate} \underset{(+H_2)}{\overset{\text{Succinic dehydrogenase } (-H_2)}{\rightleftarrows}} \text{Fumaric acid}$$

$$\text{Fumarate} \underset{(-H_2O)}{\overset{\text{Fumarase } (+H_2O)}{\rightleftarrows}} \text{Malic acid}$$

$$\text{Malate} \underset{(+\text{H}_2)}{\overset{\text{Malic dehydrogenase } (-\text{H}_2)}{\rightleftarrows}} \text{Oxalacetate}$$

$$\text{Malate} \underset{(+\text{H}_2)}{\overset{\text{Malic enzyme } (-\text{H}_2)}{\rightleftarrows}} \text{Pyruvate} + \text{CO}_2$$

The hypothesis that citric acid is formed through the condensa
2-carbon and a 4-carbon compound has been accepted by many s
This concept has been supported by research carried out with
The 2-carbon compound concerned is presumed to be aceta
4-carbon compound, a dicarboxylic acid, may be formed by a con
of two 2-carbon compounds or one 1-carbon and one 3-carbon co
Foster and Carson (1950), using labeled carbon dioxide, showe
may be incorporated into citric acid, in part, at least, through th
Werkman reaction. They also demonstrated the condensation
2-carbon compounds to form $C_4$-dicarboxylic acids by an isotopic-
method. Martin, Wilson, and Burris (1950); Martin and Wilson
Lewis and Weinhouse (1951); and Mosbach, Phares, and Carson
have confirmed the role of 1-carbon compounds in the synthesis
acid by *A. niger*. Bomstein and Johnson (1952) verified the oc
of $C_2,C_2$ condensation.

Shu, Funk, and Neish (1954) used a medium containing gluco
as the only source of carbon to study the mechanism of citric acid
tion by *A. niger*. As a result of mathematical analysis of their d
following conclusions were suggested by them: (1) 37 to 40 per cen
citric acid formed was produced from recycled $C_4$-dicarboxylic ac
40 per cent of the dicarboxylic acid was formed by a $C_2,C_2$ conde
and the remaining 60 per cent through a $C_1,C_3$ condensation; and
per cent of the glucose was dissimilated according to the scheme o
and Meyerhof and the rest through a mechanism which involved ca
labeled pyruvic acid.

## Some References concerning Citric Acid

Albritton, E. C. (ed.): Standard Values in Nutrition and Metabolism, W
*Development Center Technical Report* 52-301, December, 1953, p. 189.

Amelung, H.: Beiträge zur Säurebildung durch *Aspergillus niger*, *Zeit. physiol*
**166**: 161 (1927).

———: Wachstum und Säurebildung von *Aspergillus niger* unter Wasser, *Ch*
**54**: 118 (1930).

Bernhauer, K.: Zum Chemismus der Citronensäurebildung durch Pilze.
Säurebildung aus verschiedenen Kohlenstoffverbindungen. II. Die C
säurebildung aus Gluconsäure, *Biochem. Zeit.*, **197**: 309–342 (1928).

———: "Die oxydative Gärungen," Springer-Verlag, Berlin, Vienna, 1932.

———: "Gärungschemisches Praktikum," Springer-Verlag, Berlin, Vienna, 19

———: Biochemie der oxydativen Gärungen, *Ergeb. Enzymforsch.*, **3**: 185–27

———, N. Böckl, and H. Siebenäuger: *Biochem. Zeit.*, **253**: 37–41 (1932).

——— und A. Iglauer: Über die Säurebildung aus Zucker durch *Aspergill*
*Biochem. Zeit.*, **286**: 45 (1936).

———, J. Rauch, and G. Gross: *Biochem. Zeit.*, **319**: 499–514 (1949).

———, ———, und H. Knobloch: Über die Säurebildung aus Zucker durch
*Biochem. Zeit.*, **307**: 298–306 (1941).

ıstein. R.. and M. J. Johnson: The Mechanism of the Formation of Citrate and Oxalate by *Aspergillus niger, Jour. Biol. Chem.*, **198**: 143–153 (1952).

low, G. H., and M. J. Johnson: Effect of Aeration on Citric Acid Production in Fifty-gallon Tanks, *Ind. Eng. Chem.*, **44**: 2945–2946 (1952).

:ewitsch, W. S., et M. S. Gaewskaya: *Compt. rend. acad. sci. U.R.S.S. (N.S.)*, **3**: 405 (1935).

—, E. W. Menzschinskaja, und E. I. Trofimova: Zur Biochemischen Herkunft von ˈitronen- und Oxalsäure. I. Über die Bildung von Citronensäure aus Essigsäure, *Biochem. Zeit.*, **272**: 290 (1934). II. Die Mycelsubstanzen als ein Quelle der ˈäurebildung, *Biochem. Zeit.*, **272**: 364 (1934).

, F. J.: Citric Acid Fermentation on Solid Materials, *Ind. Eng. Chem.*, **27**: 201 1935).

enger, F.: The Production of Citric Acid by Fermentation Processes, *Ind. Chem- st.* **5**: 181–184 (1929).

—, V. Subramaniam, and T. K. Walker: The Mechanism of the Formation of ˈitric and Oxalic Acids from Sugars by *Aspergillus niger*, I, *Jour. Chem. Ind.*, anuary, 1927, pp. 200–208.

erjee, N. P.: Citric Acid from Molasses by *A. niger, Jour. Indian Chem. Soc.*, *ıd. & News Ed.*, **5**: 201–205 (1942).

szcz, T., and J. Janicki: Recent Advances in the Fermentation Industries, *Jour. oc. Chem. Ind.*, Nov. 6, 1936.

- and K. Leonhard: The Production of Citric Acid from Lactic Acid and from lcohol, *Biochem. Jour.*, **30**: 1947–1952 (1936).

- und E. Peyros: Optimale Bedingungen der Citronensäurean-häufung, sowie, nige Beobachtungen zur Theorie der Citronensäurebildung, *Biochem. Zeit.*, **30**: 325 (1935).

- und D. Tiukow: Biochemische Umbildungen der Essigsäure durch Schimmel- lze und über den Chemismus der Citronensäurebildung, *Biochem. Zeit.*, **229**: 3 (1930).

und M. Zakomorny: Biochemische Umbildung des Zuckers durch Schimmel- lze. Die Umbildung der Fumarsäure, Anhäufung der Ameisensäure und der emismus der Oxalsäurebildung, *Biochem. Zeit.*, **259**: 156 (1933).

und ———: Über den Mechanismus der Umbildung von Ameisensäure zu al- und Kohlensäure durch verschiedene Schimmelpilze, *Biochem. Zeit.*, **279**: (1935).

und ———: Über die Bedeutung der Äpfelsäure bei der Umbildung der Essig- re in Citronensäure durch verschiedene Schimmelpilze, *Biochem. Zeit.*, **285**: –355 (1936).

R., e L. Brüll: Sul meccanismo della fermentazione citrica.-1, *Ann. chim. appl.*, . 3–11 (1939).

t, M. T.: Citric Acid Fermentation of Beet Molasses by *Aspergillus niger* in merged Culture, *Can. Jour. Technol.*, **30**: 82–88 (1952).

ne, V. W.: Commercial Production of Acids by Fungi, *Econ. Botany*, **2**: 145– (1948).

J. N.: The Citric Acid Fermentation of *Aspergillus niger, Jour. Biol. Chem.*, 15–37 (1917).

ıpta, G. C., K. C. Saha, and B. C. Guha: The Fermentative Production of ric and Oxalic Acids from "Gur" and Molasses, *Science and Culture (India)*, ): 397–398 (1938).

———, and ———: The Fermentative Production of Citric and Oxalic Acids n Molasses, *Jour. Indian Chem. Soc., Ind. & News Ed.*, **3**: 64–74 (1940).

, W. P., and S. C. Prescott: Citric Acid Fermentation, *Ind. Eng. Chem.*, **26**: (1934).

F.: *Soc. Forh. Math. Naturw.*, Vol. 15, 1918–1919.

I.: Zur Theorie der Citronensäuregärung, *Biochem. Zeit.*, **275**: 373 (1935).

I. von: Grundlagen und Ergebnisse der Pflanzenchemie, Brunschweig, 1909, d III.

J. W.: "Chemical Activities of Fungi," Academic Press, Inc., New York,

——— and S. F. Carson: Citric Acid Formation by *Aspergillus niger* thr densation of $3C_2$ Moieties, *Jour. Am. Chem. Soc.*, **72**: 1865 (1950).

———, ———, S. Ruben, and M. D. Kamen: *Proc. Natl. Acad. Sci. U.* (1941).

Franzen, H., and F. Schmidt: *Ber.*, **58**: 222–226 (1925).

Gardner, J. F., L. Valerie-James, and S. D. Rubbo: Production of Citri Mutants of *Aspergillus niger*, *Jour. Gen. Microbiol.*, **14**: 228–237 (1956

Gerhardt, P., W. W. Dorrell, and I. L. Baldwin: Citric Acid Fermentati Molasses, *Jour. Bact.*, **52**(5): 555–564 (1946).

Giordani, M.: Fermentazione citrica, *Chimica e industria (Milan)*, **17**: 77–

Gray, D. J. S.: Cane Molasses Fermentations. Part IV, The Determinatio Acid in Molasses Fermentation Media, *Jour. S. African Chem. In* **2**(2): 89–93 (1949).

Gudlet, M., V. Kirsanowa, and V. Makarowa: *Schriften Wiss. Forsch. Inst. mittelind. (U.S.S.R.)*, **1**: 45 (1935).

Henneberg, W.: "Handbuch der Gärungsbakteriologie," Paul Parey, Be Vol. 1.

Herrick, H. T., and O. E. May: Fermentation as a Factor in Producing Acids for Chemical Industry, *Chem. Eng.*, **42**: 142–143 (1935).

Herzog, R. O., and A. Z. Polotsky: *Zeit. physiol. Chem.*, **59**: 125–128 (1909).

Iwanoff, N. N., and associates: *Proc. Inst. Sci. Research Food Ind. (U.S.* 3–153 (1935); **3**: 1–167 (1936).

——— and E. S. Zwetkoff: Biochemistry of Fungi, *Ann. Rev. Biochem* (1933); **5**: 585 (1936).

Karow, E. O.: The Production of Citric Acid in Submerged Culture, docto Rutgers University, New Brunswick, N.J., March, 1942.

——— and S. A. Waksman: Production of Citric Acid in Submerged Cult *Eng. Chem.*, **39**: 821–825 (1947).

Kostytschev, S., and V. Berg: Conditions of Biochemical Production of Cit *Bull. State Inst. Agr. Microbiol. (U.S.S.R.)*, **5**: 8–27 (1933).

Lamb, A. R.: *Proc. Hawaiian Sugar Planters' Assoc., Rept. Expt. Sta.*, **56**: (1936).

Lewis, K. F., and S. Weinhouse: Studies on the Mechanism of Citric Acid Pr in *Aspergillus niger*, *Jour. Am. Chem. Soc.*, **73**: 2500–2503 (1951).

Lockwood, L. B., and A. J. Moyer: The Production of Chemicals by Fila Fungi, *Botan. Rev.*, **4**: 140–164 (1938).

Martin, S. M.: The Succinoxidase System in *Aspergillus niger*, *Can. Jour. M* **1**: 6–11 (1954).

———: Effect of Ferrocyanide on Growth and Acid Production of *Aspergill* *Can. Jour. Microbiol.*, **1**: 644–652 (1955).

——— and W. R. Waters: Production of Citric Acid by Submerged Ferme *Ind. Eng. Chem.*, **44**: 2229–2233 (1952).

———, P. W. Wilson, and R. H. Burris: Citric Acid Formation from *Aspergillus niger*, *Arch. Biochem.*, **26**: 103–111 (1950).

——— and ———: Uptake of $C^{14}O_2$ by *Aspergillus niger* in the Formation Acid, *Arch. Biochem.*, **32**: 150–157 (1951).

Maze, P., and A. Perrier: *Compt. rend.*, **139**: 311–313 (1904).

Mosbach, E. H., E. F. Phares, and S. F. Carson: The Role of One-carbo pounds in Citric Acid Biosynthesis, *Arch. Biochem.*, **35**: 435–442 (1952).

Moyer, A. J.: Effect of Alcohols on the Mycological Production of Citric Surface and Submerged Culture. I. Nature of the Alcohol Effect, *Appl. biol.*, **1**: 1–7 (1953*a*).

———: II. Fermentation of Crude Carbohydrates, *Appl. Microbiol.*, **1**: 8–13

Perlman, D.: Mycological Production of Citric Acid—The Submerged Method, *Econ. Botany*, **3**: 360–374 (1949).

———, W. W. Dorrell, and M. J. Johnson: Effect of Metallic Ions on the tion of Citric Acid by *Aspergillus niger*, *Arch. Biochem.*, **10**(3): 131–143

———, D. A. Kita, and W. H. Peterson: Production of Citric Acid fro Molasses, *Arch. Biochem.*, **11**(1): 123–129 (1946).

—, H. A. Lardy, and M. J. Johnson: Determination of Citric Acid in Fermentation Media and Biological Materials, *Ind. Eng. Chem., Anal. Ed.*, **16**: 515–516 (1944).

uin, L. H. C.: "Bijdrage tot de Kennis der oxydatieve dissimilatie van *Aspergillus niger* van Tieghem," W. D. Meinema, Delft, 1938.

es, N.: *Am. Jour. Botany*, **19**: 559–567 (1932).

co, A., e A. DiCapua: Sopra l'influenza del ferro sulla fermentazione citrica dell' *Aspergillus niger, Chimica e industria (Milan)*, **14**: 289 (1932).

rick, H., and A. B. Clark: On the Mechanism of Oxalic Acid Formation by *spergillus niger, Biochem. Jour.*, **13**: 329 (1919).

akrishnan, C. V.: The Tricarboxylic Acid Cycle in *Aspergillus niger, Enzymologia*, 17 (fasc. 3): 169–174 (1954).

– and S. M. Martin: Isocitric Dehydrogenase in *Aspergillus niger, Arch. Biochem. and Biophys.*, **55**: 403–407 (1955).

–, R. Steel, and C. P. Lentz: Mechanism of Citric Acid Formation and Accumulation in *Aspergillus niger, Arch. Biochem. and Biophys.*, **55**: 270–273 (1955).

ts, O., and D. Murphy: A Rapid Fermentation Method for the Production of alcium Citrate and Calcium Gluconate from Beet Molasses, *Sci. Proc. Roy. ublin Soc.*, **23**: 307–314 (1944).

er, R.: *Biochem. Zeit.*, **202**: 131 (1928).

., A. Funk, and A. C. Neish: Mechanism of Citric Acid Formation from Glucose by *Aspergillus niger, Can. Jour. Biochem. and Physiol.*, **32**: 68–80 (1954).

and M. J. Johnson: Effect of the Composition of the Sporulation Medium on tric Acid Production by *Aspergillus niger* in Submerged Culture, *Jour. Bact.*, (2): 161–167 (1947).

and ———: Citric Acid Production by Submerged Fermentation with *Aspergillus niger, Ind. Eng. Chem.*, **40**(7): 1202–1205 (1948).

ler, J. R.: Bachelor of Science thesis, University of Wisconsin, Madison, Wis., 45.

R., C. P. Lentz, and S. M. Martin: Submerged Citric Acid Fermentation of gar Beet Molasses. Increase in Scale, *Can. Jour. Microbiol.*, **1**: 299–311 (1955).

S. M. Martin, and C. P. Lentz: A Standard Inoculum for Citric Acid Protion in Submerged Culture, *Can. Jour. Microbiol.*, **1**: 150–157 (1954).

V.: *Zentr. Bakt. Parasitenk.*, Abt. II, **82**: 321 (1930).

C., and M. B. Church: "The Aspergilli," The Williams & Wilkins Company, timore, 1926.

and J. N. Currie: Aspergillus niger Group. Oxalic-Acid Production of Species Aspergillus, *Jour. Agr. Research*, **7**: 1–15 (1916).

on, V., J. J. R. Campbell, and P. C. Trussell: The Influence of Zinc, Iron, per and Manganese on the Production of Citric Acid by *Aspergillus niger*, *r. Bact.*, **59**: 517–527 (1950).

James, L., S. D. Rubbo, and J. F. Gardner: Isolation of High Acid-yielding ants of *Aspergillus niger* by a Paper Culture Selection Technique, *Jour. Gen. robiol.*, **14**: 223–227 (1956).

secke, H. W.: A Review of Information on Mycological Citric Acid Produc- . *Chem. & Eng. News*, **23**: 1952–1959 (1945). (Contains 145 references.)

T. K.: Recent Developments in the Biochemistry of Bacteria, *Jour. Soc. m. Ind. (Trans. & Com.)*, **55**: 61T (1936).

V. Subramaniam, and F. Challenger: The Mechanism of the Formation of ic and Oxalic Acids from Sugars by *Aspergillus niger*, II, *Jour. Chem. Ind.*, , pp. 3044–3054.

. J.: BIOS Final Report No. 489, Item No. 22 (1946).

d, F. H. S., and F. Hardy: Manufacture of Calcium Citrate and Citric Acid Lime Juice, *Ind. Eng. Chem.*, **17**: 1283 (1925).

C.: "Beiträge zur Kenntnis einheimischer Pilze," Hannover und Leipsig. av Fischer, Jena, 1892.

Note sur la fermentation citrique, *Bull. soc. chim.*, **9**: 728–730 (1893).

Préparation d'acide citrique de synthèse, par la fermentation du glucose, *pt. rend.*, **117**: 332 (1893).

———: Über zwei weitere freie Citronensäure bildende Pilze, *Chem. Ztg.,* 1023 (1897).

———: *Ber.,* **58**: 2616 (1925).

Wells, P. A., and H. T. Herrick: Citric Acid Industry, *Ind. Eng. Chem.,* 3 (1938).

———, A. J. Moyer, and O. E. May: The Chemistry of the Citric Acid Fer I. The Carbon Balance, *Jour. Am. Chem. Soc.,* **58**: 555 (1936).

——— and G. E. Ward: Fermentation Processes, *Ind. Eng. Chem.,* **31**: 172

Wilson, C. P.: What Becomes of Second-grade Lemons? Citric Acid. Th an Important Chemical Industry Developed in an Attempt to Salv That Otherwise Would Be Wasted or Inefficiently Utilized, *Chem. Eng* (1923).

Wünschendorff, M., et C. Killian: Observations sur le métabolisme de *vulgaris L, Compt. rend.,* **187**: 572 (1928).

## Some Patents

Bakonyi, I.: French Patent 819,173, Oct. 12, 1937; also British Patent 474,8 9, 1937; and Hungarian Patent 117,222, Nov. 15, 1937.

Barbisan, L. E., and Soc. d'Exploitations des Procédés L. Cerini: French 833,631, Oct. 26, 1938.

Bernhauer, K.: German Patent 587819, Nov. 30, 1933.

Bleyer, B.: German Patent 434729, Oct. 6, 1926.

Boehringer, C. F., und G. M. B. H. Soehne: British Patent 452,460, Aug. 2 and German Patent 636135, Oct. 2, 1936.

Cahn, F. J.: French Patents 675,236, and 675,237, Oct. 29, 1929; also Canadian 306,081, Nov. 25, 1930; British Patent 345,368, Mar. 26, 1931; British 348,214, May 14, 1931; Production of Organic Acids by Fermentatio Patent 1,809,797, June 9, 1931; Citric Acid Manufacture, U.S. Patent 1, June 30, 1931; Citric Acid Manufacture, U.S. Patent 2,047,669, July 14, 1

Chauncey Chemical Corporation: French Patent 819,279, Oct. 13, 1937; also Patent 491,534, Sept. 5, 1938.

Distillers Company, Ltd.: British Patent 287,604, Mar. 27, 1928.

Eisenman, W., and M. D. Blumenfeld: Process for Production of Citric A Fermentation, U.S. Patent 2,364,701, Dec. 12, 1944.

Falck, R.: German Patent 426926, Mar. 29, 1926 and 473727, Mar. 20, 1929.

Fernbach, A., and J. L. Yuill: French Patents 610,121 and 610,122, May 29 also British Patents 266,414 and 266,415, Feb. 28, 1927; Process for the tion of Citric Acid, U.S. Patents 1,691,965 and 1,691,966, Nov. 20, 1928.

Filosofov, M. S.: Russian Patents 17,805, June 30, 1927, and 19,622, Mar. 31, 1

Frey, A.: German Patent 567071, Mar. 30, 1933.

Kanhäuser, F.: Process for the Production of Citric Acid by Fermentatio Patent 1,779,001, Oct. 21, 1930.

Lilly, C. H.: Process of Mold Fermentation and Apparatus Therefor, U.S. 1,936,983, Nov. 28, 1933.

Lorentz, W. K.: German Patent 679847, Aug. 15, 1939; also British Patent 5 Feb. 8, 1940.

Montan und Industrialwerke vorm. Joh. Dav. Starck: French Patent 650,072, 1929.

Nebe, E.: German Patent 698905, Nov. 19, 1940.

Nussbaum, J.: French Patent 801,273, July 31, 1936.

Peake, A. M.: Production of Citric Acid, U.S. Patent 1,788,739, Jan. 13, 1931.

Scholvien, K.: German Patent 162622, Sept. 13, 1905.

Schweiger, L. B., and R. L. Snell: Fermentation Process, U.S. Patent 2,476,159

Shmuk, A. A.: Russian Patents 30,272, July 21, 1933, and 35,187, Mar. 31, 193

Snell, R. L., and L. B. Schweiger: Production of Citric Acid by Fermentatio Patent 2,492,667, 1949.

Szücs, J.: Canadian Patent 251,180, June 30, 1925; also Austrian Patent 1 Sept. 25, 1925; German Patent 461356, June 13, 1928; Process for Pro

Citric Acid by Means of Fermentation, U.S. Patent 1,679,186, July 31, 1928; Method of Producing Citric Acid by Fermentation, U.S. Patent 2,353,771, July 18, 1944.

sman, S. A.: Process of Fermentation, U.S. Patent 2,400,143, May 14, 1946.

— and E. O. Karow: Process for the Production of Citric Acid, U.S. Patent 2,394,031, 1946.

mer, C.: French Patent 228,554, Mar. 11, 1893; also British Patent 5,620, Dec. 9, 1893; Process of Making Citric Acid, U.S. Patent 515,033, Feb. 20, 1894; and erman Patent 72957, Feb. 20, 1894.

dward, J. C., R. S. Nicholls, and R. L. Snell: Canadian Patent 422,142, Aug. 15, 1944.

rski, B.: Method of Producing Citric Acid, U.S. Patent 1,066,358, July 1, 1913.

er, J.: French Patent 803,630, Oct. 5, 1936; British Patent 462,350, Mar. 8, 1937; rocess for Producing Citric Acid, U.S. Patent 2,072,919, Mar. 9, 1937; Produc-ion of Citric Acid, U.S. Patent 2,121,064, June 21, 1938.

## e References concerning the Production of Citric Acid from Cane Molasses

d. A. E.: Cane Molasses Fermentations. Part II: An Account of the Difficulties nherent in the Citric Acid Fermentation of Cane Molasses, *S. African Ind. hemist,* March, 1949.

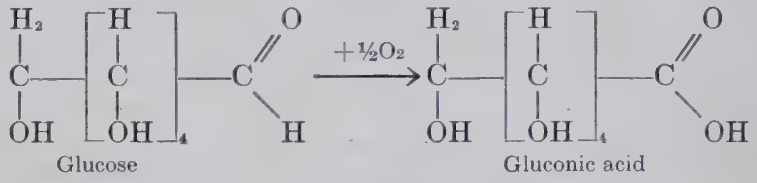
-, H. H. Sephton, and G. C. A. Van der Westhuizen: Part III. An Acount of a earch for Strains of Aspergilli That Will Give Good Yields of Citric Acid in tationary Culture from All Varieties of Natal Molasses, *Jour. S. African Chem. nst.* (*N.S.*), 2(2): 77–88 (1949*a*).

-, ———, and ———: Part V. The Influence of Inorganic Anions, Particularly hloride and Sulphate, upon the Citric-Oxalic Acid Fermentation, *Jour. S. frican Chem. Inst.* (*N.S.*), 2(2): 95–104 (1949*b*).

## CHAPTER 29

# THE GLUCONIC ACID FERMENTATION

Gluconic acid ($C_6H_{12}O_7$) is produced by the oxidation of the a
grouping of glucose. This conversion is represented by the fo
equation:



**Methods of Production.** By chemical means, gluconic acid m
prepared from glucose by oxidation with a hypochlorite solution.
conic acid may be produced, in a second method, by the electrolysi
solution of the sugar containing a measured amount of a bromide.

Gluconic acid may be produced by the fermentation of nutrient
tions of glucose by many molds and bacteria. Great differences
however, between the various microorganisms in respect to their a
ability for commercial use. Some, of course, produce only small
of gluconic acid; some may be classed as slow fermentors; some
stable cultural characteristics.

**Historical.** The production of this acid by microorganisms was
observed by Boutroux in 1878. *Mycoderma aceti* (*Acetobacter*
produced a substance from glucose which was first thought to be
acid, but which was identified two years later as gluconic acid.

Molliard, in 1922, discovered gluconic acid as a product of mold fer
tation, along with citric and oxalic acids. The acid was produc
sucrose mashes as a result of the action of *Sterigmatocystis nigra* (*A*
*gillus niger*). Molliard later developed some optimum conditions fo
fermentation.

In 1924 Bernhauer discovered a strain of *A. niger* that would pro
gluconic acid almost exclusively when in the presence of calcium ca
ate. Bernhauer has shown that thin mats grown at a low tempera
with a low supply of nitrogen favored the production of gluconic
On the other hand, higher yields of citric acid were obtained when
fermentation temperature was relatively high, the nitrogen supply
and the mats heavy.

**Preparation by Mold-Fermentation.** An extensive study of the
conic acid fermentation has been carried out over a period of year

y, Herrick, Moyer, Hellbach, Wells, Stubbs, and others of the U.S. artment of Agriculture. In 1938 it was shown by Gastrock, Porges, ls, and Moyer that gluconic acid could be successfully produced on a -plant scale from refined corn sugar (commercial glucose). In gen- gluconic acid may be produced by mold fermentation, using the ow-pan method or using submerged mold growths under increased ressure. The latter method is much superior to the former, not only spect to the time required for the fermentation but also in the yield n the ease of production.

## THE SHALLOW-PAN METHOD

the shallow-pan method, a sterile nutrient glucose solution is inocu- with the mold and incubated in cabinets designed to prevent con- ation. The mycelial mat develops as in the citric acid fermenta- and glucose is transformed to gluconic acid by the mold enzyme—an se. During incubation the mat must not be disturbed.

**ection of Pan.** In the production of gluconic acid through fermen- by molds, a very important consideration is that of the selection appropriate material for the construction of the pan. Several ements must be satisfied, namely, noncorrosiveness to acid, lack of ty, low cost, and durability. Iron, zinc, and ordinary aluminum tacked by acid. Nickel, lead, copper, and Monel metal are somewhat Glass would be satisfactory, but it is easily broken and expensive. tin and bakelite may be used, but they are also expensive. Iron, enameled, is satisfactory, but it is easily chipped, unwieldy, and ive. Lacquers are not good. Aluminum of a high purity, 99.45 per luminum, and containing less than 0.1 per cent of copper and nese has given good results.

## RODUCTION BY THE SUBMERGED-CULTURE METHOD

yer in 1928 demonstrated that agitation, aeration, and the use of carbonate increased the yield of gluconic acid from four to six hat of unaerated cultures. *Aspergillus fumaricus* was used in solutions. The yield of citric acid by this organism was not altered change in conditions. Thies (1930) used the same mold but d oxygen instead of air through the medium and obtained similar Currie, Kane, and Finlay (1933) reported yields of gluconic acid as 90 per cent when the mold growth was submerged, when the was maintained in a high degree of agitation by means of a device, and when air was drawn in large quantities into the .

**ratory Scale.** In work carried out by the Color and Farm Waste of the U.S. Department of Agriculture (May et al., 1934), glu- id was produced in high yields by submerged growths of *Peni- hrysogenum* under increased air pressure. The medium used contained 3 g. of $NaNO_3$, 0.15 g. of $KH_2PO_4$, 0.125 g. $MgSO_4 \cdot 7H_2$( equivalent of 20 per cent of pure glucose per liter. The addition calcium carbonate ($CaCO_3$) for each 4 g. of glucose caused ar in the yields of acid and likewise served as a supporting mediun

The fermentation was carried out in 500-ml. gas-washing bo sintered-glass false bottoms. In each bottle, 200 ml. of nutrien was sterilized (at 15 lb. pressure for 15 min.), cooled, and inocul mold spores. Calcium carbonate, when used, was sterilized se and added aseptically. Filtered, humidified air was permitted through the sintered-glass bottoms of the bottles at a contro (40 ml. per min.), thus providing agitation and aeration. Under going conditions, 80 to 87 per cent yields of gluconic acid were at a temperature of 30°C. when the air pressure was maint 3 atmospheres or above (in an autoclave) and calcium carbonate in the ratio indicated.

**The Rotary Drum (Laboratory Size).** In 1935, Herrick, H and May described the rotary drum, an apparatus for producing acid by submerged mold growths. The drum was developed in an to find the most suitable type of equipment for the industrial pro of gluconic acid by molds. It was constructed of aluminum co less than 0.1 per cent of manganese and copper. Buckets and baff attached to the inside shell of the drum, which served to bring the of the air into intimate contact with the nutrient glucose soluti the mold. Facilities were available for sterilizing the drum with before charging it with the medium, calcium carbonate ($CaCO$ inoculum.

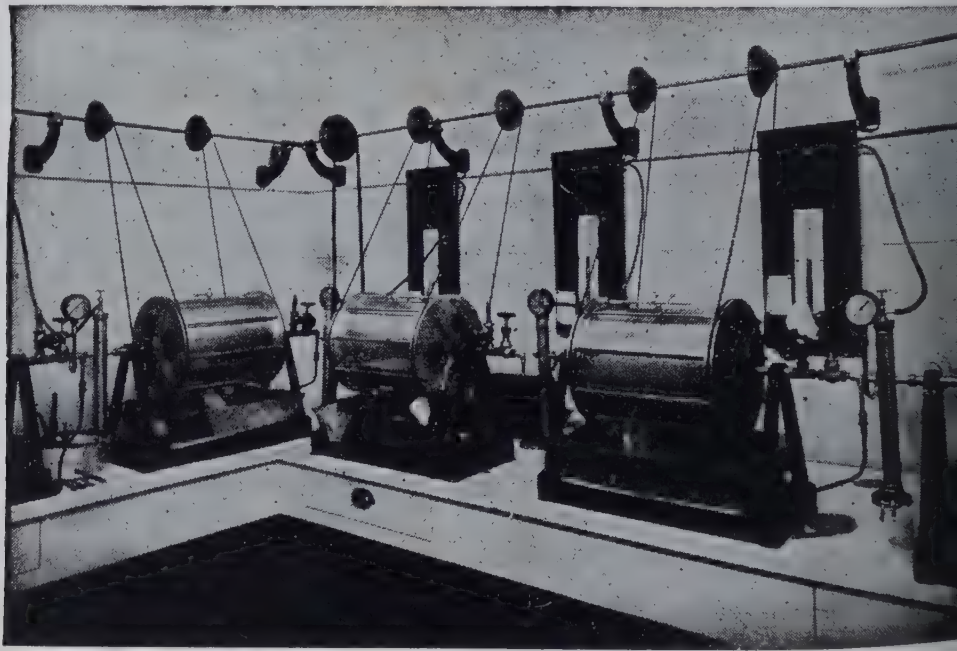


FIG. 99. Laboratory-scale fermentation apparatus. [*Courtesy of E. A. G* *N. Porges, P. A. Wells, and A. J. Moyer, Ind. Eng. Chem.*, 30: 782 (1938). *Co* 1938 *by the American Chemical Society and reprinted by permission of the co* *owner.*]

uring operation, sterilized air is introduced through an inlet and wed to accumulate until the desired pressure is built up in the drum. reafter a definite quantity of air flows into and out of the drum at a stant rate. The drum is rotated by means of belts applied to its side flanges.

he rotary drum is shown in the accompanying photograph (Fig. 99). itial experimentation with the rotary drum indicated that the time ired for the fermentation was considerably reduced by its use. le 162 compares the production of gluconic acid by several different hods.

TABLE 162. THE PRODUCTION OF GLUCONIC ACID BY DIFFERENT METHODS *

| ype of nentation | Organism | Fermentation vessel | Yield of acid (theoretical),† per cent | Fermentation period, days |
|---|---|---|---|---|
| e | *Penicillium luteum purpurogenum* | Shallow pan (aluminum) | 57.4 | 11 |
| erged essure) | *P. chrysogenum* | Glass bottle (sintered glass, false bottom) | 80.4 | 8 |
| erged ssure) | *P. chrysogenum* | Rotary drum (aluminum) | 80.0 | 2.2 |

A. Wells, A. J. Moyer, J. J. Stubbs, H. T. Herrick, and O. E. May, *Ind. Eng.* 29: 653 (1937). Copyright 1937 by the American Chemical Society and re- d by permission of the copyright holder.

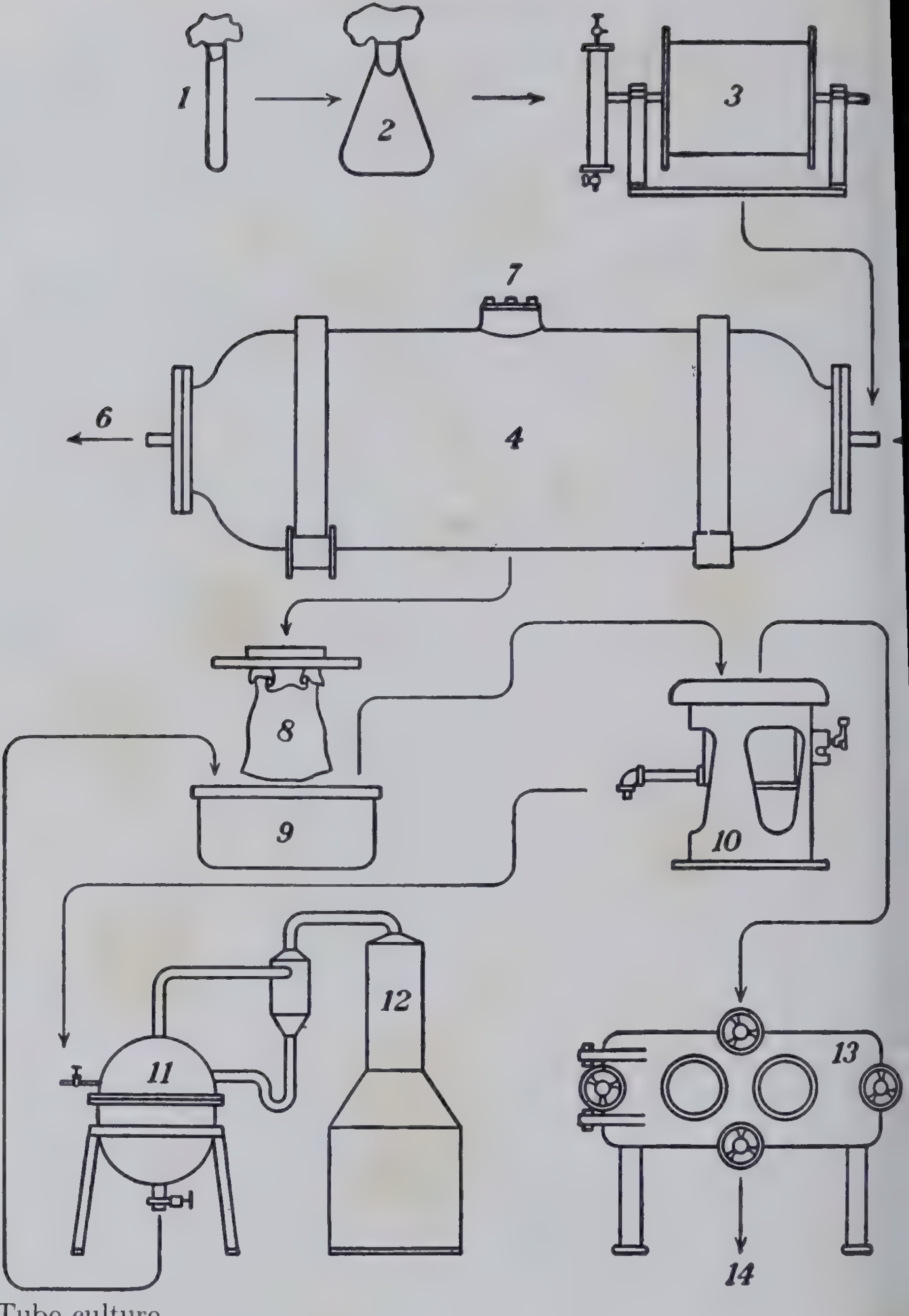
om 20 per cent glucose solutions.

*ails of the Rotary-drum Process (Laboratory Scale).* In the rotary- process, the selection of an appropriate organism, the maintenance optimum oxygen supply, the composition of the various media ved, and the use of calcium carbonate in the process are impor-

ORGANISM. *P. chrysogenum*, an organism used in producing glu- cid by submerged growths under pressure, does not readily produce rge quantities of spores required for inoculating mashes. *A. niger* 67 of the Industrial Farm Products Research Division) was d by Wells, Moyer, and their associates because it possessed certain ble characteristics—it readily produced spores and uniform tations.

OXYGEN SUPPLY. The effectiveness of the oxygen supply in the drum process is controlled by three factors: agitation, air flow, and ssure. Upon the efficiency of these factors depends, largely, the of the fermentation, provided the medium and inoculum are tory.

num results were obtained when the rotation of the drum (see was 13 r.p.m. Higher speeds produced excessive frothing of the



1. Tube culture.
2. Sporulation flasks.
3. Small rotating aluminum germination drums (17-liter volume).
4. Large rotating aluminum drum fermentor (540-liter volume).
5. Air inlet; water for fermentation solution and inoculum from 3 are duced here.
6. Air outlet.
7. Handhole; commercial dextrose, nutrients, and sterile calcium carb added here.
8. Bag filter.
9. Aluminum tank for neutralization and crystallization; calcium hydroxid is added here.
10. Centrifuge (stainless-steel basket, aluminum-lined curb).
11. Vacuum evaporator for mother liquors.
12. Condenser.
13. Vacuum drier.
14. To calcium gluconate

FIG. 100. Flow sheet of gluconic acid production. [*Courtesy of E. A. N. Porges, P. A. Wells, and A. J. Moyer, Ind. Eng. Chem.*, **30**: 782 (1938). 1938 *by the American Chemical Society and reprinted by permission of right owner.*]

lium. Air flows of 400 to 1,200 ml. per min. for a volume of 3,200 ml. nash were satisfactory. (Costs increase as the amount of air used eases.) By maintaining the gauge at pressures of 30 to 45 lb. per in., excellent results were obtained. A pressure of 30 lb. per sq. in. considered most practical, however, since higher pressures increased danger of damaging the equipment and producing leaks. Table 163 trates the effect of air pressure on the fermentation of glucose to onic acid by *A. niger:*

ABLE 163. THE EFFECT OF VARIOUS AIR PRESSURES ON GLUCONIC ACID YIELDS*

Air flow, 1,200 ml. per min.; speed, 13 r.p.m.; volume of medium, 3,200 ml.; lucose available, 495 g.; fermentation period, 18 hr.; temperature, 30°C.)

| pressure, sq. in., sq. cm.) | Glucose consumed, g. | Gluconic acid produced, g. | Gluconic acid yield based on | |
|---|---|---|---|---|
| | | | Glucose consumed, per cent | Glucose available, per cent |
| (0.35) | 178 | 173 | 89.1 | 32.1 |
| (1.05) | 257 | 258 | 92.1 | 47.9 |
| (2.11) | 336 | 351 | 93.0 | 65.1 |
| (3.16) | 429 | 454 | 97.1 | 84.2 |

A. Wells, A. J. Moyer, J. J. Stubbs, H. T. Herrick, and O. E. May, *Ind. Eng.* . 29: 653 (1937). Copyright 1937 by the American Chemical Society and ed by permission of the copyright owner.

IA. The effect of variations in the nature and quantities of the nt salts and other substances and in the amount of glucose on the obtained with a given organism is well illustrated in the gluconic rmentation. A medium of one composition is used for maintaining owth of the culture, a second medium for inducing sporulation, a nedium for stimulating germination, and a fourth medium for the tion of gluconic acid. Inasmuch as the media used for the produc- gluconic acid on a pilot-plant scale vary but slightly from those or the preparation of the acid on a laboratory scale, a summary former is given in Table 164 (courtesy of the authors and edi-

**hnique of Preparing Inoculum.** *A. niger*, strain 67, is cultured for at 30°C. on slants of medium A. These slant cultures are used to te at least twenty 1-liter Erlenmeyer flasks, each containing 150 ml. ium B. The flasks are incubated at 30°C. for 7 days, and then the earing mycelium is aseptically transferred to a vessel containing s of medium C, where it is thoroughly broken up by means of a ical agitator. The contents of the flask are divided into two por- nd each is placed in a small rotary drum—the total charge in each ccupying approximately one-third of its total volume. The follow- litions are maintained during germination:

| | |
|---|---|
| Air pressure | 30 lb./sq. in. gauge (155-mm. |
| Air flow | 375 ml./liter/min. |
| Speed of rotation | 5.8 r.p.m. |
| Temperature | 30°C. |
| Time | 24 hr. |

TABLE 164. SUMMARY OF MEDIA USED FOR GLUCONIC ACID PRODUCTION
*gillus niger* *

| Ingredient | A, culture | B, sporulation | C, germination |
|---|---|---|---|
| G./liter: | | | |
| Refined corn sugar † | 30.0 | 50.0 | 100.0 |
| $MgSO_4 \cdot 7H_2O$ | 0.10 | 0.12 | 0.25 |
| $KH_2PO_4$ | 0.12 | 0.144 | 0.30 |
| $(NH_4)_2HPO_4$ | None | 0.56 | 0.80 |
| $NH_4NO_3$ | 0.225 | None | None |
| Peptone | 0.25 | 0.20 | 0.02 |
| Potatoes | 200 | None | None |
| Agar | 20.0 | 1.5 | None |
| $CaCO_3$ | 4.0 | None | 37.5 ‡ |
| Beer, ml./liter | None | 45 | 40 |
| Kind of water | Distilled | Distilled | Tap |

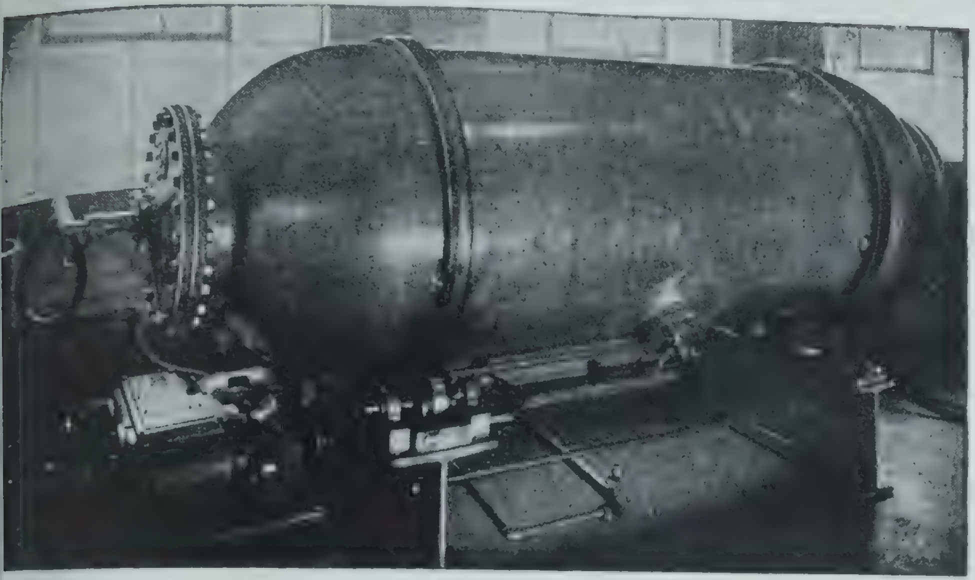
* E. A. Gastrock, N. Porges, P. A. Wells, and A. J. Moyer, *Ind. Eng. Ch*
782 (1938). Copyright 1938 by the American Chemical Society and reprinted
mission of the copyright owner.

† Refined corn sugar, containing 91.5 per cent of dextrose and correspondin
to dextrose monohydrate, was used in almost all the research.

‡ Separately sterilized.

By permitting the spores to germinate in medium C, the fermen
period is shortened and less inoculum is required for a given volu
fermentation medium D.

**The Pilot-plant Fermentor.** The solutions containing the germ
spores are transferred aseptically to a large-scale fermentor (Fig
containing medium D. Wells, Lynch, Herrick, and May (1937)
described this fermentor. The drum is 3 by 6 ft. and is construc
aluminum sheet, containing 99.5 per cent aluminum and less than (
cent of copper, iron, and manganese. End castings are made
aluminum-silicon alloy. The tank holds 420 gal., but ordinarily
140 gal. of charge is added—larger charges increase the fermen
time. Buckets and baffles are welded to the interior of the shell and
tion as in the small rotary fermentors. Facilities are provided for st
ing the drum and its contents. An aluminum pipe, which is attac
the drum's interior surface and looped back and forth around the
of the buckets and baffles, extends around one-third of the periph
the drum on the portion opposite the handhole. By connecting one
the tubing to a steam line and the other to a drain, steam may be su
for sterilization purposes (Fig. 102). Means are available for fillin



01. Large-scale fermentation apparatus. [*Courtesy of E. A. Gastrock, N.* *, P. A. Wells, and A. J. Moyer, Ind. Eng. Chem.,* 30: 782 (1938). *Copyright* 1938 *American Chemical Society and reprinted by permission of the copyright* ]

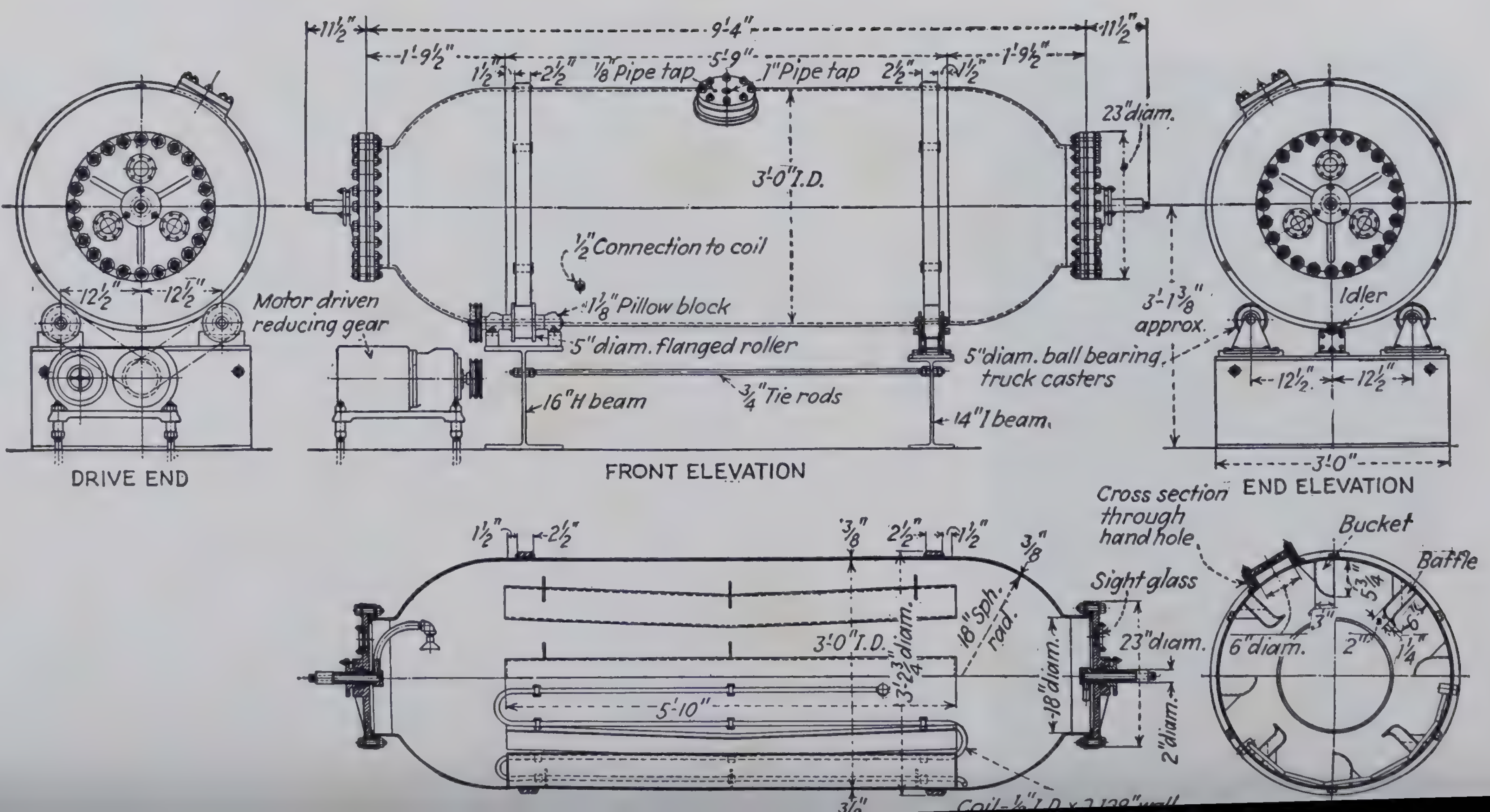
ing the drum. Sterile, humidified air is passed through the drum pressure. For further details, the reader is referred to the papers in the foregoing paragraphs.

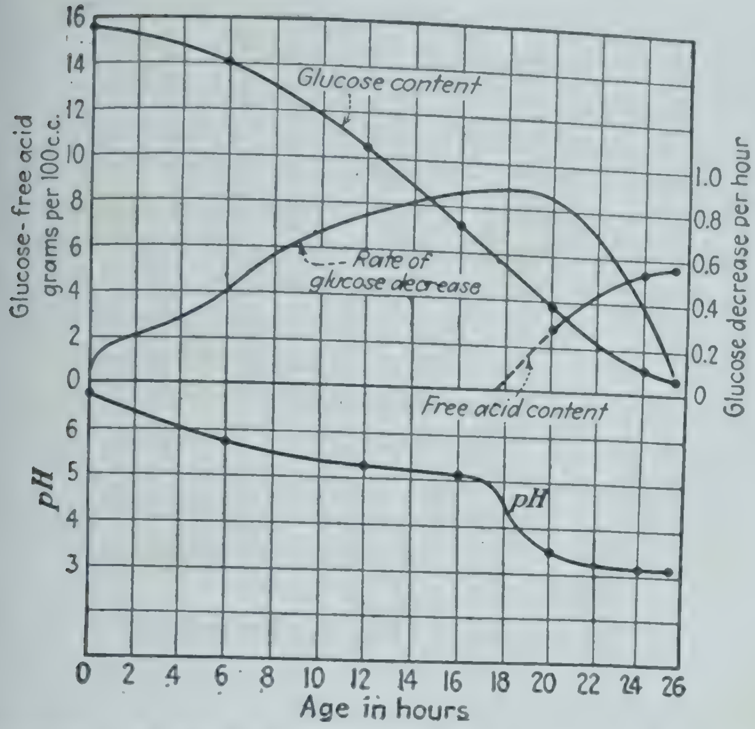
**utralization of Gluconic Acid.** High acidity inhibits the fermen- . The rate at which glucose is converted to gluconic acid is more in the presence of undissolved calcium carbonate than in the pres- f free acid. It has been shown by Gastrock and his associates that of 26 g. of calcium carbonate per liter of fermentation medium ctorily controls the pH of the medium. This quantity of calcium ate dissolves readily in the medium and is equivalent to 102.1 g. of ic acid per liter. The use of an excess of calcium carbonate retards mentation, since the calcium gluconate formed tends to crystallize l prevent free contact of the medium with the mold.

greatest activity in the medium coincides quite closely with the t which free acid develops. Until the free acid develops, the pH medium remains close to 5.5; it then drops rapidly to a value of ess. Free acid, with the drop in pH, usually appears about 17 hr. he start of the fermentation. During the drop in pH, the activity s diminished. Thus a pH of 5.5 is favorable for the production acid, since the maximum rate of conversion is at pH values of than 5. Figure 103 correlates pH with glucose utilization.

**continuous Production of Gluconic Acid.** A process has been ed by Porges and his associates (1940) in which gluconic acid may luced successfully on a semicontinuous basis and which possesses advantages over the single-batch method previously used.

oxidations were carried out in laboratory- and semi-plant-size ors, which have already been described, under the following ns: an air pressure of 30 lb. per sq. in. (gauge), an air flow of


11½"
9'-4"
11½"
1'-9½"
5'-9"
1'-9½"
1½"
2½"
⅛" Pipe tap
1" Pipe tap
2½"
1½"
23" diam.
3'-0" I.D.
½" Connection to coil
1⅛" Pillow block
5" diam. flanged roller
5" diam. ball bearing truck casters
¾" Tie rods
16" H beam
14" I beam
Motor driven reducing gear
12½"
12½"
Idler
3'-1⅜" approx.
12½"
12½"
3'-0"
DRIVE END
FRONT ELEVATION
END ELEVATION
Cross section through hand hole
Bucket
Baffle
Sight glass
23" diam.
18" diam.
2" diam.
18" Sph. rad.
3'-0" I.D.
3'-2¾" diam.
5'-10"
⅜"
⅜"
1½"
2½"
2½"
1½"
5¼"
6"
3"
2"
1¼"
6" diam.



3. Correlation of pH with utilization of glucose (15.6 g. per 100 ml.) by
*llus niger* at 6.0 r.p.m. [*Courtesy of E. A. Gastrock, N. Porges, P. A. Wells,*
*J. Moyer, Ind. Eng. Chem.,* 30: 782 (1938). *Copyright* 1938 *by the American*
*al Society and reprinted by permission of the copyright owner.*]

. per liter of medium per minute, a rotation speed of 9.5 r.p.m., and
erature of 30°C.

result of research, a glucose concentration of approximately 11.5 g.
ml. was found to be optimum, rather than one of 15 g. per 100 ml.
of lower glucose concentrations resulted in shorter fermentation
, but in lowered efficiencies of production.

initial charge of nutrient medium in a fermentor was inoculated
pregerminated culture. The inoculum for each subsequent charge
ured by floating the mycelia from the previously completely
ed medium. A fermentation was judged to be complete when less
er cent of glucose remained unconverted or when the rate of acid
ion was negligible in comparison to that of active fermentation.
n was accomplished by reducing the pressure in the fermentor to
the atmosphere for a period of 35 min. During this interval most
nycelia rose to the upper portions of the medium. Subsequently
er 80 per cent of the charge was removed, and a new charge of
was introduced. The upper 20 per cent of the charge retained
previous fermentation contained most of the active mycelia—
n 85 per cent.

the foregoing conditions, a fermentation (excluding the initial
usually complete in a little more than 9 hr.

ny as 13 successive fermentations have been carried out, using
eral procedure, with no apparent loss in efficiency, according to
nd his associates.

Later Porges, Clark, and Aronovsky (1941) showed that th of *A. niger* 67 could be recovered by pressure filtration and nine successive fermentations of media containing 16 g. of glu about 2.6 g. of calcium carbonate per 100 ml. The pressure filter structed of aluminum since this metal was nontoxic to *A. niger* inhibitory to the fermentation. Previous studies had shown tha of an iron filter was unsatisfactory because of the inhibitory ef sumably caused by the iron and other metals dissolved by the acid and held by the mycelia. The use of centrifugal filtration w to be a more efficient method for separating the mycelia from mented liquor in those cases where the concentration of free acid

The advantages of this process are the elimination of the la found in the single-batch method through the repeated use of the the elimination of the necessity for preparing the pregerminated i for all except the initial charge, and the maintenance of a high oxidation of glucose to gluconic acid.

**Fermentation of Concentrated Glucose Solutions.** There are advantages to fermenting glucose solutions of relatively high co tion to gluconic acid. However, prior to studies by Moyer, Um and Stubbs (1940), satisfactory production of gluconic acid from trated glucose solutions by semicontinuous methods using rotary was impractical because of one or both of two controlling facto precipitation of calcium gluconate and the formation of free glucon The precipitation of calcium gluconate immediately inhibits the f tation. Although the solubility of calcium gluconate at 30°C. about 4 g. per 100 ml., no precipitation takes place normally unti 15 g. per 100 ml. is present, owing in part to the formation of a sup rated solution. Thus, glucose solutions producing yields of calciu conate of less than 13 to 15 g. per 100 ml. may be completely neut with calcium carbonate without precipitation of the calcium salt.

On the other hand, if the free gluconic acid is not neutraliz fermentation capacity of the mold may be inhibited after pro exposure to the acid. In the semicontinuous production of gluconi prolonged exposure of the mycelium to the acid increases the lag in subsequent fermentations wherein the mycelium is reused. In so containing 20 to 30 per cent of glucose, the fermentation rate is dec during the final phase owing to the inhibitory effect of the acid, at time the pH may be reduced to 3 to 3.5.

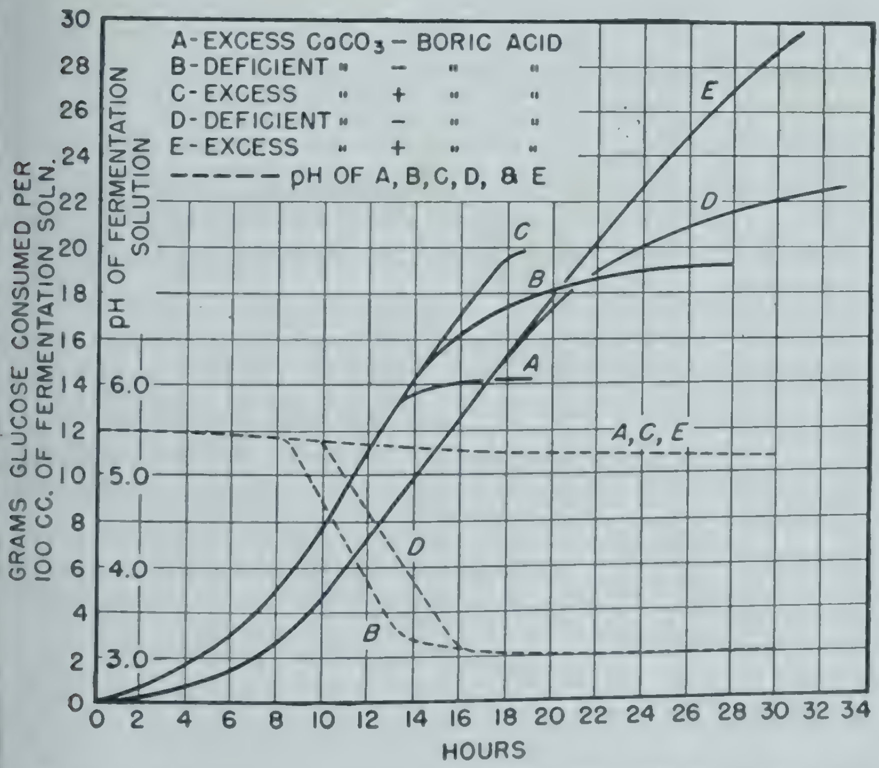
In 1940, Moyer, Umberger, and Stubbs found that it was poss ferment 20 to 35 per cent glucose solutions, in about one-half of th previously reported, by the use of boric acid, borax, or other boron pounds, in the presence of an excess of calcium carbonate. Small q ties of the boron compounds prevented the precipitation of calciu conate, while the excess of calcium carbonate prevented the format free gluconic acid. Moyer and his associates demonstrated that th cipitation of the calcium gluconate formed during the normal fermen of 20, 25, 30, and 35 per cent glucose solutions could be prevent the use of 500, 1,000, 1,500, and 2,500 p.p.m. of boron, respectively.

using boron compounds, it is necessary to employ concentrations lie within limits tolerated by the mold and that do not interfere with ecovery of crystalline calcium gluconate from aqueous solutions. It ascertained that *A. niger* 67 was less tolerant of boric acid than *A.* 3, which also was an excellent producer of gluconic acid by sub- d-culture methods. *A. niger* 67 was strongly inhibited in the pres- of more than 400 p.p.m., but *A. niger* 3 grew well in the presence of p.p.m. of boron.

optimum time for the addition of the boron compounds was after 8 g. of glucose per 100 ml. had been consumed. Fermentation was ted when the boron compounds were added at the time of inocula- In order to prevent a violent evolution of carbon dioxide, foaming of edium, and an appreciable increase in the volume of the fermentor, ric acid, or borax, was added in the dry form.

effect of a deficiency or excess of calcium carbonate, with or with- oric acid, upon the fermentation of glucose to gluconic acid by *er* 3 is shown in Fig. 104.

conic acid production on a semicontinuous basis by *A. niger* 3 from nt solutions containing 25 g. of glucose per 100 ml., a slight excess cium carbonate, and boric acid (1,500 p.p.m. boron) is illustrated 105.



Effect of deficiency or excess of calcium carbonate with and without boric ie fermentation of glucose to gluconic acid by *Aspergillus niger* 3. Deficiency n carbonate, 2.34 g. per 100 ml.; *A*, *B*, and *C*, 20 g. glucose per 100 ml. at and *E*, 30 g. per 100 ml. at start; *C*, 500 p.p.m. boron; *E*, 1,500 p.p.m. boron. *of A. J. Moyer, E. J. Umberger, and J. J. Stubbs, Ind. Eng. Chem.*, 32: 0). *Copyright* 1940 *by the American Chemical Society and reprinted by n of the copyright owner.*]

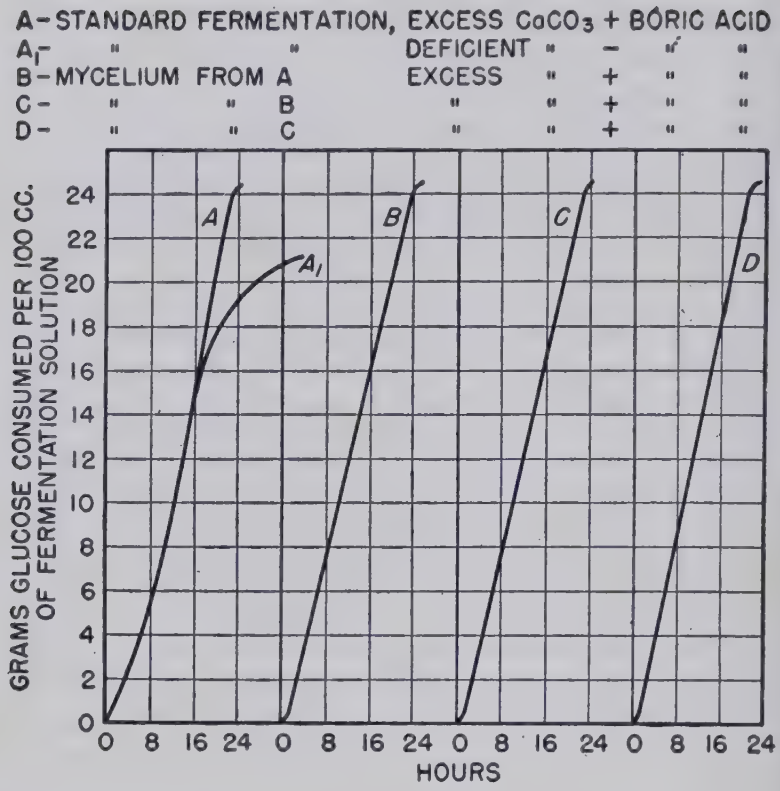


Fig. 105. Gluconic acid production from glucose by repeated reuse of *As*
*niger* 3 mycelium. [*Courtesy of A. J. Moyer, E. J. Umberger, and J. J. Stub*
*Eng. Chem.*, 32: 1379 (1940). *Copyright* 1940 *by the American Chemical Soc*
*reprinted by permission of the copyright owner.*]

**Industrial Production.** The production of gluconic acid on an
trial basis from glucose solutions containing nutrient salts and c
carbonate and using *A. niger* has also been described by Williams (

**Production of Sodium Gluconate.** A method for producing s
gluconate by a submerged process, using *A. niger* NRRL 3, ha
described by Blom and associates (1952). The medium for the
plant fermentation (Table 165) is sterilized at 121°C. and at a pH
for 15 to 30 min., cooled, adjusted to pH 6.5 with sterile NaOH, anc
ulated with either a spore suspension or a 24-hr. vegetative growth
mold. An air pressure of 20 to 30 lb. per sq. in. is maintained with
fermentor, and the mash is aerated with 1 volume of air per volu
medium per minute or more. Foaming is controlled by the addition
required amount of 1 per cent octadecanol in 95 per cent ethanol
temperature is kept at 92 to 94°F. and the pH at 6.5 ± 0.5 with N
The duration of the fermentation is usually under 40 hr. for glucose
centrations up to 35 per cent. The glucose consumption rate, aft
initial lag, is about 1.5 per cent per hour until 0.1 per cent of
remains. After the fermentation is complete, the liquor is dec
filtered, and evaporated to 42 to 45 per cent solids. The pH of the co
trate is adjusted to 7.5 with NaOH. The solids are then drum-drie
ground to 100-mesh size. Figure 106 shows a flow sheet for the pr

The inoculum is prepared for the fermentation medium as a spore
pension or as a vegetative growth. In the former method, stock cul

TABLE 165. SOME MEDIA USED FOR SODIUM GLUCONATE PRODUCTION *

| Ingredient | Stock-culture medium | Sporulation medium | Germination medium | Fermentation medium |
|---|---|---|---|---|
| e (commercial), g... | 30.0 | 50.0 | 10,000.0 | 136,400 |
| $\cdot 7H_2O$, g. | 0.1 | 0.12 | 31.0 | 94 |
| $)_4$, g. | 0.12 | 0.15 | 38.0 | 113 |
| $_2HPO_4$, g. | ..... | 0.60 | 80.0 | 240 |
| . | ..... | 0.20 | | |
| $)_3$, g. | 0.25 | | | |
| . g. | 4.0 | | | |
| trate, g. | ..... | 0.01 | | |
| extract, ml.† | 500+ | 30.0 | | |
| nl. | ..... | 30.0 | | |
| eep liquor, ml. | ..... | ..... | 750.0 | 2,100 |
| . | ..... | ..... | 20.0 | 60 |
| , g. | 0.25 | | | |
| . | 25.0 | 1.5 | | |
| to pH 6.5, g. | ..... | ..... | 25.0 | |
| o pH 4.5, ml. | ..... | ..... | ..... | 100 |
| (tap) to make liters | 1.0 | 1.0 | 170.3 ‡ | 568 § |
| ation time at 121°C., | 30.0 | 30.0 | 30.0 | 15–30 |

piled from data published by R. H. Blom et al., *Ind. Eng. Chem.*, **44**: 435 Copyright 1952 by the American Chemical Society and reprinted by permis- the copyright owner.

hundred g. of peeled and sliced potatoes autoclaved for 15 min. in 500 ml. ater and filtered through cheesecloth.

sterilization.

inoculation.

re maintained on the stock-culture medium (refer to Table 165), l to inoculate ten to twenty 1-liter flasks, each of which contains of sterilized sporulation medium. The flasks are incubated at or about 7 days, after which the spore-bearing mycelia are l and suspended in 10 liters of sterile water which contains 0.01 of a wetting agent (sodium lauryl sulfate is satisfactory). The aspension thus prepared is used to inoculate the fermentation . In the latter method, spores from two 1-liter flasks, prepared ated above, are suspended in 2 liters of sterile water (containing r cent of wetting agent) and the entire suspension blown into (170.3 liters) of the germination medium (refer to Table 165). erated at the rate of 0.2 volume of air per volume of medium per while the temperature is held at 33°C. A portion (usually about nt) of a 24-hr. growth of the germinated mold is used to inoculate entation medium.

eral, Blom et al. found that good results were obtained when ilated medium contained 24 to 30 per cent of corn sugar, 0.4 per orn-steep liquor (as received), 0.02 per cent of $MgSO_4 \cdot 7H_2O$,

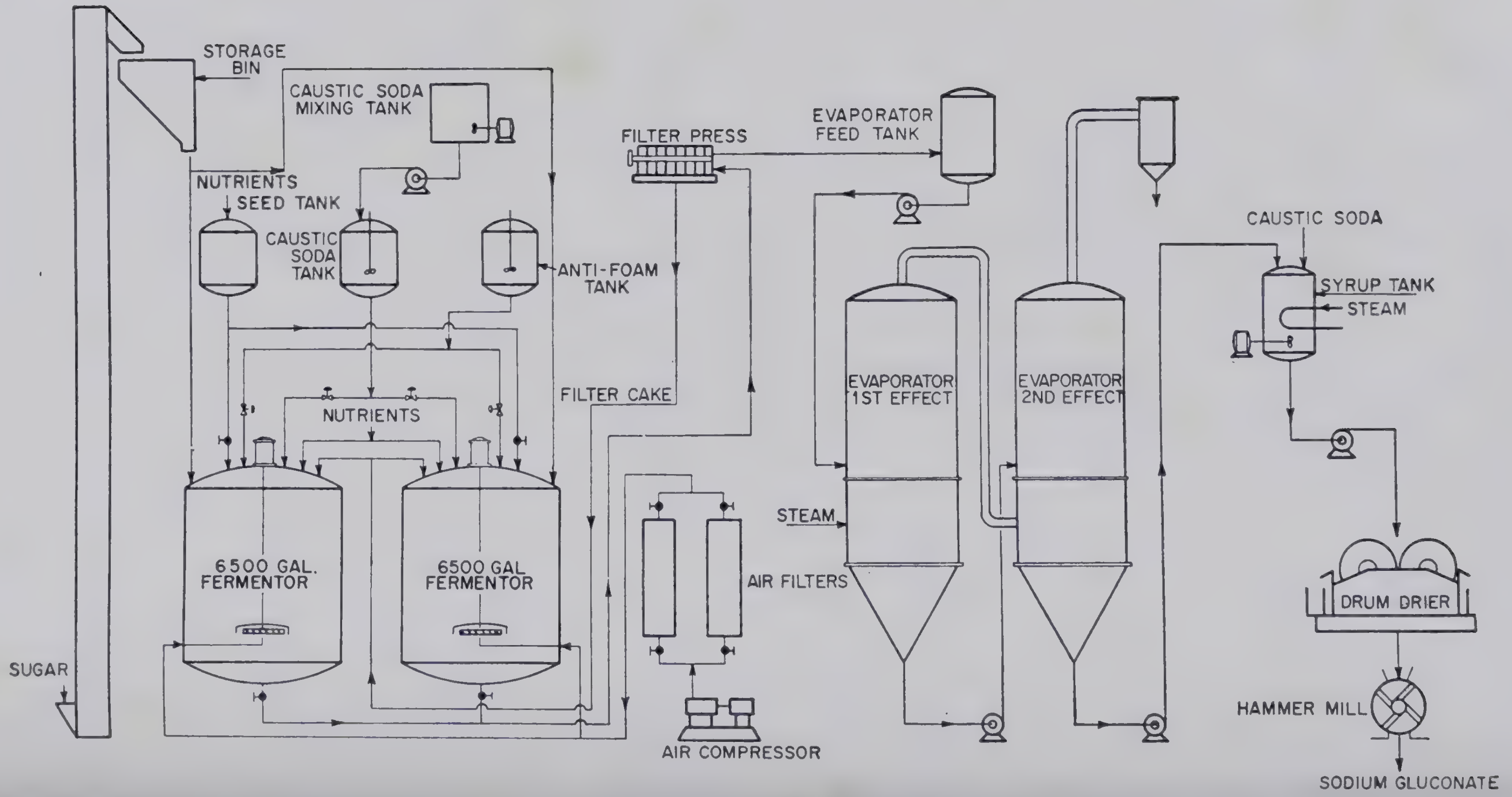

STORAGE BIN
CAUSTIC SODA MIXING TANK
NUTRIENTS
SEED TANK
CAUSTIC SODA TANK
ANTI-FOAM TANK
FILTER PRESS
EVAPORATOR FEED TANK
CAUSTIC SODA
SYRUP TANK
STEAM
NUTRIENTS
FILTER CAKE
EVAPORATOR 1ST EFFECT
EVAPORATOR 2ND EFFECT
STEAM
6500 GAL. FERMENTOR
6500 GAL FERMENTOR
AIR FILTERS
DRUM DRIER
SUGAR
HAMMER MILL
AIR COMPRESSOR
SODIUM GLUCONATE

Fig. 106. Flow sheet for production of sodium gluconate. [*Courtesy of R. H. Blom, V. F.*

| | % | | | fermentation | products | products, % | Reference |
|---|---|---|---|---|---|---|---|
| *Acetobacter gluconicum* | 5 | 30 | Aeration | 4 days | Gluconic acid | 71.8–77.2 | Porges, N., T. F. Clark, and E. A. Gastrock: *Iowa State Coll. Jour. Sci.*, **16**: 451 (1942). |
| *Penicillium luteum purpurogenum* var. *rubrisclerotium* | 20–25 | 25 | Surface | 9 days | Gluconic acid | 55–65 | Herrick, H. T., and O. E. May: *Jour. Biol. Chem.*, **77**: 185 (1928). |
| *P. chrysogenum* | 20–25 | 30 | Surface | 8–10 days | Gluconic acid and calcium gluconate | 60 | Moyer, A. J., O. E. May, and H. T. Herrick: *Zentr. Bakt. Parasitenk.*, Abt. II, **95**: 311 (1936). |
| *P. chrysogenum* | 20 | 30 | Submerged (pressure) | 8 days | Gluconic acid and calcium gluconate | 80–87 | May, O. E., H. T. Herrick, A. J. Moyer, and P. A. Wells: *Ind. Eng. Chem.*, **26**: 575 (1934). |
| *P. chrysogenum* | 20 | 30 | Submerged (pressure) | 2.2 days | Gluconic acid and calcium gluconate | 80 | Wells, P. A., A. J. Moyer, J. J. Stubbs, H. T. Herrick, and O. E. May: *Ind. Eng. Chem.*, **29**: 653 (1937). |
| *Aspergillus niger* NRRL 67 | 15.5 | 30 | Submerged (pressure) | 18 hr. | Gluconic acid and calcium gluconate | 97.1 * | Wells, P. A., A. J. Moyer, J. J. Stubbs, H. T. Herrick and O. E. May: *Ind. Eng. Chem.*, **29**: 653 (1937). |
| *A. niger* NRRL 67 | 11.5 | 30 | Submerged (pressure) | 9 hr. | Gluconic acid and calcium gluconate | ..... | Porges, N., T. F. Clark, and E. A. Gastrock: *Ind. Eng. Chem.*, **32**: 107 (1940). |
| *A. niger* NRRL 3 | 24–30 | 33.0–34.4 | Submerged (pressure) | Under 40 hr. | Sodium gluconate | | Blom, R. H., et al.: *Ind. Eng. Chem.*, **44**: 435 (1952). |

* Based on glucose consumed.

0.02 per cent of $KH_2PO_4$, 0.01 per cent of urea, and 0.04 p
$(NH_4)_2SO_4$. Soybean oil and lard oil, though satisfactory for
foam formation, decreased the sugar utilization to an impracti

**Production Data.** Some data relating to the production o
acid and gluconates are presented in Table 166.

**Uses of Gluconic Acid and Its Derivatives.** These have been
by Prescott and associates (1952). In general, gluconic acid and
atives may be used in the pharmaceutical, food, feed, textile,
industries. Calcium and "trace" elements may be introduced int
through the use of salts of gluconic acid. Ferrous gluconate an
phosphogluconate are used chemotherapeutically. Glucono-δ-lac
good leavening agent. Gluconic acid is nontoxic, noncorrosive, a
sequestering agent, when in alkaline media, for iron, alumin
copper. Sodium gluconate is also a good sequestering agent.
acid is used in the dairy industry as an acid cleaner to prevent th
tion of milkstone and as a component of milk-can cleaners. I
used in metal-cleaning compounds. It is used in textile printing, i
processing, and in photography. Gluconic acid, glucono-δ-lact
ammonium gluconate are used as acid catalysts or acid-forming c

The reader is referred to the article by Prescott et al. for additio

## References on Gluconic Acid Fermentation

Amelung, H.: Beiträge zur Säurebildung durch *Aspergillus niger, Zeit. physi*
**166**: 161 (1927).
Angeletti, A.: Azione di alcuni funghi su soluzioni di aldosi. Nota III. Fo
di acido d-gluconico per mezzo del *Penicillium crustaceum* (L.) Fries, *A*
*appl.*, **22**: 59 (1932).
——— e C. F. Cerruti. Sull'azione di alcuni funghi su soluzioni di aldosi, *A*
*appl.*, **20**: 424 (1930).
——— e L. Merlo: *Ann. chim. appl.*, **24**: 468 (1934).
Bernhauer, K.: Zum Problem der Säurebildung durch *Aspergillus niger* (
Mitteilung), *Biochem. Zeit.*, **153**: 517 (1924).
———: Über die Säurebildung durch *Aspergillus niger*. I. Allgemeines und
isches bei der Untersuchung der Säurebildungsvorgänge, *Biochem. Z*
296 (1926).
———: Über die Charakterisierung der Stämme von *Aspergillus niger* auf Gr
biochemischen Verhaltens. I. Mitteilung. Vergleichende Untersuchun
die Säurebildung durch verschiedene Pilzstämme, *Biochem. Zeit.*, **197**: 27
———: Über die Säurebildung durch *Aspergillus niger*. IV. Mitteilung. Di
tung der Mycelentwicklung für die Säurebildung, *Biochem. Zeit.*,
(1928).
———: "Die Oxydative Gärungen," Springer-Verlag, Berlin, Vienna, 1932.
———: "Gärungschemisches Praktikum," 2d ed., Springer-Verlag, Berlin,
1939.
——— und B. Gorlick: Oxydationen mittels Essigbakterien. IV. Über die
von 2-Keto-gluconsäure durch *Bact. gluconicum, Biochem. Zeit.*, **280**: 36
——— und K. Irrgang: Oxydationen mittels Essigbakterien. III. Über die
einer reduzierenden Zuckercarbonsäure ("Aldehydgluconsäure") nebe
gluconsäure, *Biochem. Zeit.*, **280**: 360 (1935).
——— und K. Schön: Oxydationen mittels *Bacterium xylinum*. II. Über die
von Glucon- und 5-Ketogluconsäure, *Zeit. physiol. Chem.*, **180**: 232 (1
Birkinshaw, J. H.: Biochemistry of the Lower Fungi, *Biol. Revs.*, **12**: 357 (1
——— and H. Raistrick: Studies in the Biochemistry of Micro-organisms

The Products of Glucose Metabolism Formed by Various Species of Fungi, *rans. Roy. Soc. London, Ser. B.*, **220**: 331 (1931).

. R. H., V. F. Pfeifer, A. J. Moyer, D. H. Traufler, H. F. Conway, C. K. Crocker, . E. Farison, and D. V. Hannibal: Sodium Gluconate Production, *Ind. Eng. hem.*, **44**: 435–440 (1952).

oux, L.: Sur une fermentation nouvelle du glucose, *Compt. rend.*, **91**: 236 1880).

-: Sur l'acide gluconique, *Compt. rend.*, **104**: 369 (1887).

ı, A. J.: The Chemical Action of Pure Cultivations of *Bacterium aceti*, *Jour. hem. Soc.*, **49**: 172 (1886).

witsch, W.: Über die Bildung der Glucon- und Citronensäure in den Pilzkulren auf Zucker, *Biochem. Zeit.*, **154**: 177 (1924).

: Über die Säurebildung bei den Pilzen, *Biochem. Zeit.*, **182**: 99 (1927).

. K. R.: Survey of the Biochemical Activities of the Acetic Acid Bacteria, emical Research, Special Report 2, H.M. Stationery Office, London, 1936.

zez, T., und D. Tiukow: Über die Säurebildung der Penicilliumarten (Link), *ochem. Zeit.*, **204**: 106 (1929).

und ———: Die Stärkebildung bei den Schimmelpilzen (*Penicillium* Link), auch ihr Zusammenhang mit der Säurebildung, *Biochem. Zeit.*, **207**: 39 (1929).

und ———: Der Zusammenhang der Stärkebildung mit der Säuerenanhäufung i den Schimmelpilzen (*Penicillium*), *Biochem. Zeit.*, **222**: 243 (1930).

R., und S. N. Kapur: Über Gluconsäure—Bildung durch Fadenpilze, *Ber.*, **B**: 920 (1924).

C., J. L. St. John, and H. W. Gerritz: Calcium Gluconate from Juice of Cull l Surplus Apples, *Ind. Eng. Chem.*, **28**: 75 (1936).

k, E. A., and N. Porges: Gluconic Acid Production on Pilot-plant Scale—ect of Variables on Production by Submerged Mold Growths, *Ind. Eng. Chem.*, 782 (1938).

M.: *Deut. Essigind.*, **33**: 385 (1929).

erg, W.: *Deut. Essigind.*, **2**: 145 (1898).

m, S.: *Bacterium gluconicum*, ein in der sogenannten Kombucha (japanischer indischer Teepilz) vorkommender Spaltpilz, *Biochem. Zeit.*, **205**: 297 (1929).

Über die Bildung von Gluconsäure und Ketogluconsäure durch *Bacterium onicum*, *Bacterium xylinum* und *Bacterium xylinoides*, *Biochem. Zeit.*, **214**: (1929).

nd P. Neuschul: Zur Biochemie der Essigbakterien zugleich ein Vorschlag eine neue Systematik, *Biochem. Zeit.*, **233**: 129 (1931).

nd ———: *Zentr. Bakt. Parasitenk.*, Abt. II, **93**: 25 (1935).

H. T., R. Hellbach, and O. E. May: Apparatus for the Application of Subed Mold Fermentations under Pressure, *Ind. Eng. Chem.*, **27**: 681 (1935).

d O. E. May: The Production of Gluconic Acid by the *Penicillium luteumurogenum* Group. II. Some Optimal Conditions for Acid Formation, *Jour. Chem.*, **77**: 185 (1928).

und Z. Lenart: Vereinfachtes Verfahren zur Darstellung von Gluconsäure, *deut. Zucker-Ind.*, **69**: 122 (1919).

vssojeva, E.: Über die Bildung von Gluconsäure durch *Aspergillus niger*, *hem. Zeit.*, **266**: 337 (1933).

hew, S., and W. Tschesnokov: *Compt. rend. acad. sci. U.R.S.S.*, **13**: 195 7).

l. L. B., and A. J. Moyer: The Production of Chemicals by Filamentous i, *Botan. Rev.*, **4**: 140 (1938).

E., and H. T. Herrick: The Production of Organic Acids by Fermentation, *Dept. Agr., Circ.* 216, May, 1932.

d ———: Some Practical and Theoretical Aspects of Mold Metabolism, *Bact.*, **28**: 145 (1934).

——, A. J. Moyer, and R. Hellbach: Semi-plant Production of Gluconic by Mold Fermentation, *Ind. Eng. Chem.*, **21**: 1198 (1929).

——, ———, and P. A. Wells: Gluconic Acid. Production by Submerged Growths under Increased Air Pressure, *Ind. Eng. Chem.*, **26**: 575 (1934).

———, ———, C. Thom, and M. B. Church: The Production of Glucon the *Penicillium luteum-purpurogenum* Group, I, *Jour. Biol. Chem* (1927).

Molliard, M.: Sur une nouvelle fermentation acide produite par le *Sterig* *nigra, Compt. rend.,* **174**: 881 (1922).

———: Nouvelles recherches sur la formation d'acides organiques par le *S* *cystis nigra* en milieux déséquilibres, *Compt. rend.,* **178**: 41 (1924).

Moyer, A. J., O. E. May, and H. T. Herrick: Production of Gluconic Acid *cillium chrysogenum, Zentr. Bakt. Parasitenk.,* Abt. II, **95**: 311 (1936).

———, E. J. Umberger, and J. J. Stubbs: Fermentation of Concentrated So Glucose to Gluconic Acid, *Ind. Eng. Chem.,* **32**: 1379–1383 (1940).

———, P. A. Wells, J. J. Stubbs, H. T. Herrick, and O. E. May: Gluc Production. Development of Inoculum and Composition of Fermentat tion for Gluconic Acid Production by Submerged Mold Growths under Air Pressure, *Ind. Eng. Chem.,* **29**: 777 (1937).

Müller, D.: Studien über ein neues Enzym Glykoseoxydase, I, *Biochem. Z* 136 (1928).

———: Studien über ein neues Enzym Glykoseoxydase, II, *Biochem. Zeit.,* (1929).

Porges, N., T. F. Clark, and S. I. Aronovsky: Gluconic Acid Production—I Recovery and Re-use of Submerged *Aspergillus niger* by Filtration, *I* *Chem.,* **33**: 1065–1067 (1941).

———, ———, and E. A. Gastrock: Gluconic Acid Production. Repeated Submerged *Aspergillus niger* for Semicontinuous Production, *Ind. Eng* **32**: 107–111 (1940).

Prescott, F. J., J. K. Shaw, J. P. Bilello, and G. O. Cragwall: Gluconic Acid Derivatives, *Ind. Eng. Chem.,* **45**: 338–342 (1952).

Schreyer, R.: Vergleichende Untersuchungen über die Bildung von Gluc durch Schimmelpilze, *Biochem. Zeit.,* **240**: 295 (1931).

Sumiki, Y.: *Bull. Agr. Chem. Soc. Japan,* **5**: 10 (1929).

———: *Jour. Agr. Chem. Soc. Japan,* **6**: 17, 23 (1930).

Takahashi, T., and T. Asai: On Gluconic Acid Fermentation. I. On *Ba* *Hoshigaki* var. *rosea* nov. spec., *Zentr. Bakt. Parasitenk.,* Abt. II, **82**: 390

——— and ———: *Jour. Agr. Chem. Soc. Japan,* **7**: 1 (1931); **8**: 703 (1932).

——— and ———: On Gluconic Acid Fermentation. III. On *Bacterium H* var. *glucuronicum* I, II, & III, nov. spec., *Zentr. Bakt. Parasitenk.,* Abt. 385 (1933).

Tamiya, H., und T. Hida: Vergleichende Studien über die Säurebildung, die A die Oxydasereaktion und das Dehydrierungsvermögen von Aspergillusarte *Phytochim. Japan,* **4**: 343 (1928–1929).

Tanaka, K.: Zur Physiologie der Essigbakterien. I. Über die Gluconsäuregär Essigbakterien, *Acta Phytochim. Japan,* **7**: 265 (1933).

Thies, W.: *Zentr. Bakt. Parasitenk.,* Abt. II, **82**: 321 (1930).

Visser't Hooft, F.: Biochemische Onderzoekingen over het geslacht Acet W. D. Meinema, Delft, 1925.

Wehmer, C.: Abnahme des Säuerungsvermögens und Änderung der Säure be Pilz. (Gluconsäure-statt Fumarsäure-Gärung), *Biochem. Zeit.,* **197**: 418

Wells, P. A., D. F. J. Lynch, H. T. Herrick, and O. E. May: Translating Mo mentation Research to Pilot Plant Operation, *Chem. Eng.,* **44**: 188 (1937)

———, A. J. Moyer, J. J. Stubbs, H. T. Herrick, and O. E. May: Gluconic Ac duction. Effect of Pressure, Air Flow, and Agitation on Gluconic Acid I tion by Submerged Mold Growths, *Ind. Eng. Chem.,* **29**: 653 (1937).

Williams, A. E.: Production of Gluconic Acid, *Mfg. Chemist,* **16**: 239–241 (19

### Some Patents

Bernhauer, K.: French Patent 707,614, 1931.

——— and L. Schulhof: Production of Gluconic Acid, U.S. Patent 1,849,053. M 1932.

. J. N., and R. H. Carter: Production of Gluconic Acid, U.S. Patent 1,896,811, b. 7, 1933.

. J. H. Kane, and A. Finlay: Process for Producing Gluconic Acid by Fungi, S. Patent 1,893,819, Jan. 10, 1903; also British Patent 370,039, 1930.

R.: German Patent 553238, 1924.

: German Patent 556761, 1931.

: German Patent 587819, 1933.

nn, S.: German Patent 522147, 1931.

Austrian Patent 127,373, 1932.

Austrian Patent 133,139, 1933.

., H. T., and O. E. May: Process for the Manufacture of Gluconic Acid, U.S. ent 1,726,067, Aug. 27, 1929.

ack, R., and W. R. Giles: Process for the Preparation of Gluconic Acid and Lactones, U.S. Patent 1,942,660, Jan. 9, 1934.

.: Process for the Oxidation of Aldoses, U.S. Patent 1,648,368, Nov. 8, 1927.

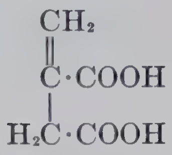
and W. Kussmaul: Oxidation of Aldoses, U.S. Patent 1,703,755, Feb. 26, 1929.

shi, T.: Process for Preparing Gluconic Acid, U.S. Patent 1,953,694, Apr. 3, 4.

## CHAPTER 30

# THE ITACONIC ACID FERMENTATION

Itaconic acid, or methylene succinic acid, has the following st formula:



$$\begin{array}{l} CH_2 \\ \| \\ C\cdot COOH \\ | \\ H_2C\cdot COOH \end{array}$$

It is an unsaturated dicarboxylic acid. The conjugated doubl the two carboxylic groups, and the allylic methylene group make i acid a reactive and versatile compound. Esters and salts are formed from it. Its esters may be polymerized alone or copolym with other monomers. Dimethyl itaconate and dibutyl itaconate a esters prepared from it which copolymerize readily with a number o monomers, such as acrylonitrile, the methacrylates, styrene, but vinyl chloride, and vinylidine chloride. The anhydride is readily f copolymers of it with vinyl acetate are useful in the condition soil.

Pure itaconic acid occurs as a white crystalline powder with a n point of 165 to 166°C. and a specific gravity of 1.632. It is soluble in at 20°C. to the extent of 8.3 g. per 100 ml. and at 80°C. to the ext 72.5 g. per 100 ml. It is insoluble in paraffinic hydrocarbons but po increased solubilities in alcohols and ketones. It is relatively nont

The first reports on the production of itaconic acid by mold fer tion were made by Kinoshita (1929, 1931, 1937), who stated that it acid and mannitol were the principal products formed from sucr an organism that he designated as *Aspergillus itaconicus* Kin Calam, Oxford, and Raistrick (1939) announced that a strain of *reus* Thom produced significant amounts of itaconic acid from g However, other strains of the same species failed to produce this ac

Extensive research on a laboratory and pilot-plant scale has carried out in the Fermentation Division, Northern Utilization Re and Development Division, U.S. Department of Agriculture (re Table 167). Kane, Finlay, and Amann (1945); Vincenty and ass (1948, 1950); Van der Westhuizen et al. (1951); and others hav carried out studies relating to the production of itaconic acid by m of fermentation.

**Organisms Employed.** Itaconic acid may be produced by c strains of *A. terreus* and *A. itaconicus*. Calam and his associates only 1 strain of *A. terreus* out of 5 examined was able to produce iic acid from a Czapek-Dox medium containing 5 per cent glucose .2 per cent potassium nitrate. Moyer and Coghill (1945) surveyed airs of this mold for itaconic acid production and obtained "promis- elds" from 1 only. This strain, designated as *A. terreus* NRRL 265, ced 28 to 29 g. of itaconic acid per 100 g. of glucose in 10 to 12 days optimum conditions. Lockwood and Reeves (1945) surveyed 308 s of *A. terreus*, which had been isolated by Raper and Alexander, of orthern Utilization Research and Development Division, and found t gave yields from glucose greater than 45 per cent of the theoreti- ne of the best of these, *A. terreus* NRRL 1960, was used in the hes of Lockwood and Reeves and others (refer to Table 167).

racteristics of ultraviolet-induced mutations of *A. terreus* have escribed by Raper and coworkers (1945) and by Lockwood, Raper, , and Coghill (1945).

k cultures of *A. terreus* may be carried on slants of Czapek-Dox n agar or of a malt agar containing 2.5 per cent malt extract, 0.1 per eptone, 2 per cent dextrose, and 2 per cent agar (Pfeifer et al.,

**ıods of Production.** Itaconic acid may be produced by surface- merged-culture methods of fermentation, by the dehydration or is of citric acid, or by the decarboxylation of aconitic acid. It is d industrially by a submerged-culture process of fermentation.

*Production.* The production of large crops of spores is necessary inoculation of flasks, pans, or seed tanks. Some of the methods cultivating large numbers of spores are described below.

r and Coghill (1945) used a liquid sporulation medium of the g composition:

| | | | |
|---|---|---|---|
| ..................... | 10.0 g. | Corn-steep liquor ........... | 5.0 ml. |
| ..................... | 5.0 g. | Fe tartrate .................. | 0.005 g. |
| ..................... | 0.060 g. | $CuSO_4 \cdot 5H_2O$ ............... | 0.004 g. |
| $I_2O$ .............. | 0.050 g. | $MnSO_4 \cdot 4H_2O$ ................ | 0.005 g. |
| ..................... | 0.100 g. | Agar ........................ | 0.100 g. |
| ..................... | 5.00 g. | Distilled water to ........... | 1.0 liter |
| ..................... | 3.00 g. | | |

edium was dispensed in 200-ml. Erlenmeyer flasks in amounts of ıch and sterilized. Good yields of spores were obtained in 5 to 7 27°C.

other type of sporulation medium described by Moyer and 25 g. of wheat bran was moistened with 20 ml. of the medium above. The moistened wheat bran was placed in 750-ml. Erlen- sks and sterilized. The cooled product was then inoculated with d incubated for 5 to 7 days, during which time a heavy crop was produced. The bran was allowed to dry, and 2 volumes of ıole-wheat flour were added and thoroughly mixed by means of spatula. According to Moyer and Coghill, about 0.20 g. of this taining mixture was adequate for seeding a 200-ml. Erlenmeyer about 0.5 g. was sufficient for a 3-liter Fernbach flask.

TABLE 167. DATA CO

| Investigators | Lockwood and Reeves | Moyer and Coghill | Lockwood and Ward | Lo<br>a |
|---|---|---|---|---|
| Reference | *Arch. Biochem.*, **6**: 455 (1945). | *Arch. Biochem.*, **7**: 167 (1945). | *Ind. Eng. Chem.*, **37**: 405 (1945). | *Ar*<br>*c*<br>3 |
| Nature of fermentation | Surface | Surface | Surface | Su<br>(a |
| Strain of *Aspergillus terreus used* | NRRL 1960 | NRRL 265 | NRRL 1960 | N |
| Sugar, per cent | Glucose<br>25 | Glucose<br>25 | Glucose<br>16.5 | |
| Principal source of nitrogen, g./liter | $NH_4NO_3$<br>2.5 | $NH_4NO_3$<br>2 | $NH_4NO_3$<br>2.5 | N |
| Amount $MgSO_4 \cdot 7H_2O$, g./liter | 4.5–5 | 0.25 | 4.4 | |
| Corn-steep liquor, ml./liter | 4 | 4 | 4 | |
| pH | 1.9–2.3 | 1.8–2 | ca. 2 | 1 |
| Acid used for adjusting pH | $HNO_3$ | $HNO_3$ | $HNO_3$ | H |
| Temperature | 30°C. | 30°C. | 30–32°C. | 3 |
| Fermentor size | 200 ml. | 200 ml. | 12 liters | 30 |
| Material of construction | Glass | Glass | Aluminum | G |
| Aeration, vol./vol. medium/min. | ...... | ...... | ...... | .. |
| Air pressure, p.s.i.g. | ...... | ...... | ...... | .. |
| Agitation, r.p.m. | ...... | ...... | ...... | .. |
| Antifoam agent | ...... | ...... | ...... | .. |
| Inoculum | Spores | Spore–wheat-flour mixture | Spores | 1<br>pell<br>125 |
| Time of fermentation | 12 days | 10–12 days | 12 days | 12 |
| Yield, % approx. max. | ca. 37 * | 28–29 * | 30 † | 25. |

* Based on sugar consumed. † Based on sugar supplied.

Lockwood and Ward used a sporulation medium of the follo composition in the semi-pilot-plant-scale production of itaconic acid:

| | |
|---|---|
| Glucose monohydrate (commercial) | 275 g. |
| $NaNO_3$ | 5 g. |
| $MgSO_4 \cdot 7H_2O$ | 0.024 g. |
| KCl | 0.005 g. |
| $H_3PO_4$ | 0.003 g. |
| Concentrated corn-steep liquor | 0.5 ml. |
| Distilled water to | 1,000 ml. |

TACONIC ACID FERMENTATION

| nty.<br>adis,<br>al. | Vincenty et al. | Van der Westhuizen et al. | Nelson et al. | Pfeifer et al. | Pfeifer et al. |
|---|---|---|---|---|---|
| *ol.*<br>*m.*<br>*rto Rico,*<br>6. | *El Crisol,* 4 : 51 (1950). | *Jour. Appl. Chem.,* 1 : 365 (1951). | *Ind. Eng. Chem.,* 44 : 1166 (1952). | *Ind. Eng. Chem.,* 44 : 2975 (1952). | U.S. Patent 2,657,173, Oct. 27, 1953. |
| merged | Surface | Surface | Submerged | Submerged | Submerged |
| .C.C.<br>020 | A.T.C.C. 10020 and NRRL 1960 | A.T.C.C. 10029 and NRRL 1960 | NRRL 1960 | NRRL 1960 | |
| crose<br>8 | Sucrose<br>14–17 | Sucrose<br>12–18 | Glucose<br>6 | Glucose<br>6 | Glucose<br>5–7 |
| $NO_3$<br>.5 | $NH_4NO_3$<br>2.5 | $NH_4NO_3$<br>5–8 | $(NH_4)_2SO_4$<br>2.67 | $(NH_4)_2SO_4$<br>2.7 | $(NH_4)_2SO_4$<br>3 |
| .5 | 4.4 | 6–9 | 5 | 0.8 | 0.8 |
| .5 | 4 | ...... | 1.5 | 1.8 | 1.5 |
| 1.9 | 1.8–2.3 | 3 | 1.8–2 | 2.1–4.9 | 2.5–5 |
| $SO_4$ | $H_2SO_4$ | $H_2SO_4$, HCl, or $HNO_3$ | $H_2SO_4$ | $H_2SO_4$ or itaconic | $H_2SO_4$ or itaconic |
| 1°C. | 30–31°C. | 30°C. | 34°C. | 95°F. | 93–95°F. |
| ters | 6 liters | 230 ml. | 20 liters | 300 or 600 gal. | |
| ass | Glass | Glass | Stainless steel No. 360 | Stainless steel No. 302 or 347 | |
| t 1 | ...... | ...... | 1/30 | 1/8 | 1/4 |
| .. | ...... | ...... | 15 | 10–20 | 15 |
| .. | ...... | ...... | ...... | 75–175 | 100–125 |
| . | ...... | ...... | Octadecanol | 0.75% octadecanol in ethanol | 1% octadecanol in ethanol |
| for<br>rs | Spores | Spores | 0.8% by volume | 5–10% by volume | 5–10% by volume |
| ys | 12 days | 3 weeks | 4–6 days | 2–3 days | 66–97 hr. |
| | 41 † | ca. 32 †, ‡ | 45–54 † | 60† | 61–65 † |

. 10029.

nedium was dispensed in 200-ml. Erlenmeyer flasks in 50-ml. and sterilized. The spores from a 10-day-old slant culture were l to seed each flask heavily. The flasks were incubated at 30°C. s, which was ample time for the production of an abundance of Lockwood and Ward stated that the culture in one flask was to inoculate 100 liters of fermentation medium.

, Vojnovich, and Heger (1952) inoculated Kolle flasks, containalt medium described above, with *A. terreus* NRRL 1960 and incubated them at 30°C. (86°F.) for 2 weeks in order to ol abundant supply of spores. The spores from an area of about 3 were used to inoculate 750 ml. of sterile inoculum medium cent glucose monohydrate, 0.27 per cent $(NH_4)_2SO_4$, 0.08 p $MgSO_4 \cdot 7H_2O$, and 0.18 per cent corn-steep liquor] in a small fla contents of the flask were aerated and maintained at 93°F. (33.8 48 hr. and then used to inoculate 20 to 40 gal. of sterile mediun same composition in a seed tank. The contents of this tank we bated at 93°F. for 48 hr. at atmospheric pressure while being aerat 0.5 to 1 volume of air per volume of medium per minute. This inoc sufficient in volume to seed 200- to 400-gal. fermentations.

**Production in Submerged Culture.** Research concerning the i tion of itaconic acid in submerged culture has been carried out by Finlay, and Amann (1945); Lockwood and Nelson (1946); Vincent (1950); Nelson et al. (1952); Pfeifer et al. (1952, 1953); and Data concerning some of these researches are summarized in Tab It will be noted that the yields of itaconic acid obtained by Lockwo Nelson and by Vincenty and associates were 25.5 and 38 per respectively, based on the weight of sugar supplied. Yields of ab per cent were obtained by Kane and coworkers. By 1952, Nelso associates were able to increase the weight yields to 54 per cen reduce the fermentation time from 8–12 days to 4 days. Pfeifer increased the yields to 60 to 65 per cent and shortened the fermen time to 2 to 3 days.

Data concerning the pilot-plant production of itaconic acid in 300 600-gal. fermentors, which included studies relating to sterili methods, medium composition, inoculum, aeration, agitation, pre and temperature, were reported by Pfeifer, Vojnovich, and Heger (1 Changes occurring in the fermentation medium are shown in Fig. 10 flow sheet for itaconic acid production by fermentation is show Fig. 108.

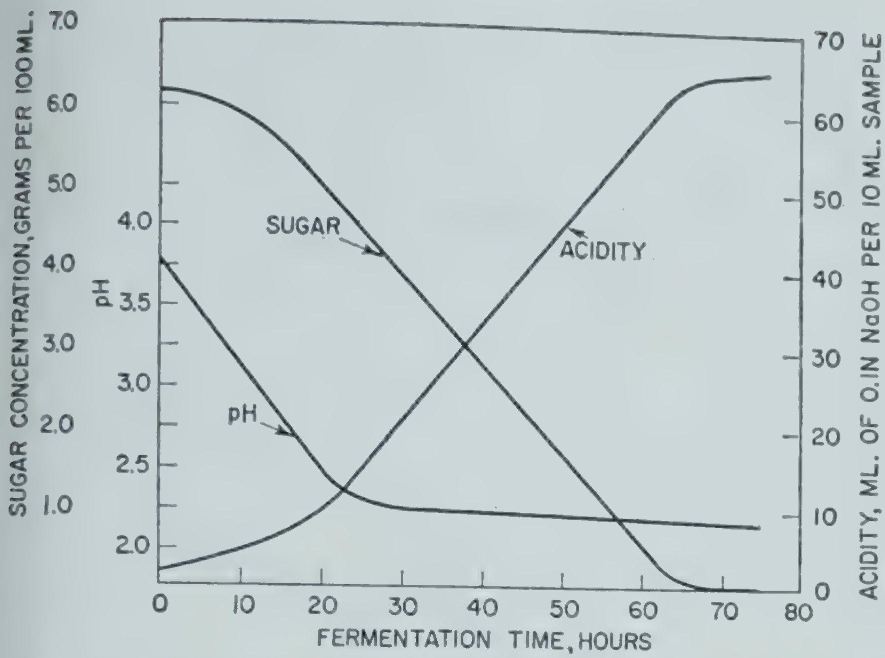
*Method of Pfeifer, Nelson, Vojnovich, and Lockwood.* Pfeifer (1953) patented a submerged process for the production of itaconic in which special emphasis was placed on the use of (1) a controlled concentration of not over 7 per cent; (2) an initial pH lower th adjusted preferably with itaconic acid; and (3) a rather large inocu

Strains of *A. terreus* or *A. itaconicus* may be used for production.

A medium of the following composition may be used for building u inoculum and for production purposes:

| | Per Cent |
|---|---|
| Corn sugar (hydrated) | 6.60 |
| Corn-steep liquor (40–45% solids) | 0.15 |
| Ammonium sulfate | 0.30 |
| Magnesium sulfate heptahydrate | 0.08 |

The pH of the medium should be adjusted before sterilization to below by the use of itaconic or sulfuric acid. Itaconic acid is prefe but sulfuric or other mineral acids may be used for economic reasons. pH may be initially adjusted to between 2.5 and 5.0.



)7. Changes in medium during fermentation. [*Courtesy of V. F. Pfeifer, novich, E. N. Heger, Ind. Eng. Chem.*, 44: 2775 (1952). *Copyright* 1952 *by the an Chemical Society and reprinted by permission of the copyright owner.*]

cose is the carbohydrate of choice, but sucrose, maltose, cane or beet ses, hydrol, dextrose sirups, or hydrolyzed starches may be used as rbon source.

concentration of sugar used exerts a critical influence on the yield e rate of acid accumulation. The concentration of fermentable ydrate should be under 7 per cent and over 5 per cent.

mber of nitrogen sources are suitable, but ammonium sulfate gives sults with corn-steep liquor.

ilding up the inoculum, the mold should be grown for 2 days at 3°F. with agitation and aeration. The inoculum should represent 5 per cent and preferably 10 per cent of the volume of the medium eeded.

erature, aeration, pressure, agitation, and foam should be con- during fermentation. The temperature is best around 93 to 95°F. satisfactory in the range of 90 to 96°F. Aeration may be varied 1 to 0.25 volume of air per volume of medium per minute. Although t essential to use air under pressure, such use is conducive to opti- ields. A pressure of 15 p.s.i.g. is advocated. An agitation rate of 125 r.p.m. was used by Pfeifer and associates; however, the rate ll depend on the design and size of the fermentor. Foam may be ed automatically through the use of 1 per cent octadecanol in

It is not necessary to adjust the pH during fermentation.

yields of 85 to 90 per cent of the theoretical may be obtained a 3-day fermentation. Weight yields of 61 to 65 per cent are

from a number of experiments are presented in Table 168.

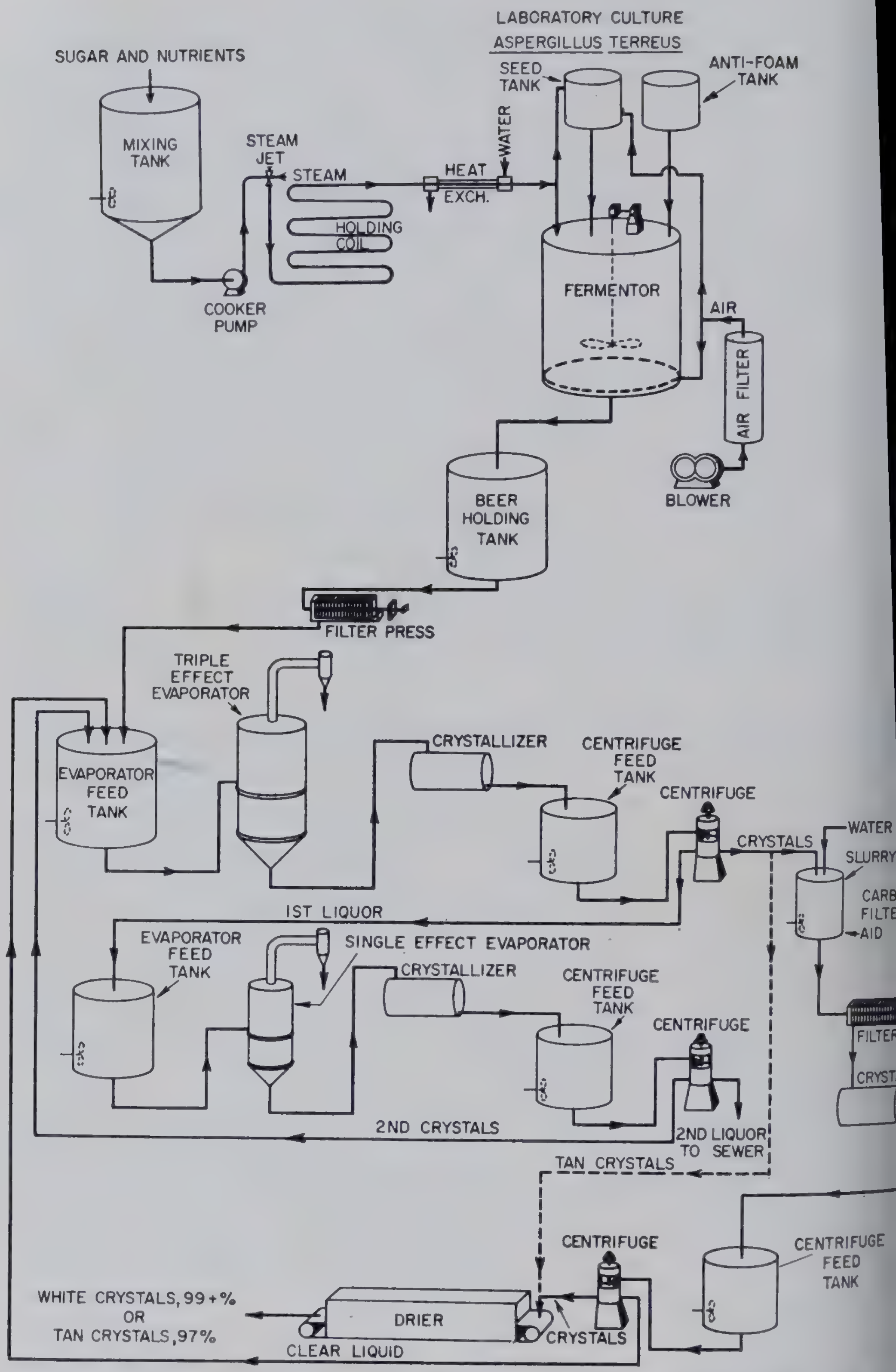

LABORATORY CULTURE
ASPERGILLUS TERREUS
SUGAR AND NUTRIENTS
SEED TANK
ANTI-FOAM TANK
MIXING TANK
STEAM JET
STEAM
WATER
HEAT EXCH.
HOLDING COIL
FERMENTOR
COOKER PUMP
AIR
AIR FILTER
BLOWER
BEER HOLDING TANK
FILTER PRESS
TRIPLE EFFECT EVAPORATOR
EVAPORATOR FEED TANK
CRYSTALLIZER
CENTRIFUGE FEED TANK
CENTRIFUGE
CRYSTALS
WATER
SLURRY
CARB
FILTE
AID
1ST LIQUOR
EVAPORATOR FEED TANK
SINGLE EFFECT EVAPORATOR
CRYSTALLIZER
CENTRIFUGE FEED TANK
CENTRIFUGE
FILTER
CRYST
2ND CRYSTALS
2ND LIQUOR TO SEWER
TAN CRYSTALS
CENTRIFUGE
CENTRIFUGE FEED TANK
WHITE CRYSTALS, 99+%
OR
TAN CRYSTALS, 97%
DRIER
CRYSTALS
CLEAR LIQUID

Fig. 108. Flow sheet for itaconic acid production by fermentation. [*Courte*
*V. F. Pfeifer, C. Vojnovich, E. N. Heger, Ind. Eng. Chem.*, **44**: 2775 (1952). C
*right* 1952 *by the American Chemical Society and reprinted by permission o*
*copyright owner.*]

TABLE 168. DATA CONCERNING SUBMERGED ITACONIC ACID FERMENTATIONS *

| eri- nt . | Sugar, % | Inoculum, % | Fermentation period, hr. | Rate of formation † | Yield, % |  | Original pH before inoculation | Acid used for adjustment |
|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  | Alkali titration | Bromine |  |  |
|  | 6.03 | 1 | 97 | 0.83 | 62.5 | 58.8 | 4.0–5.0 | $H_2SO_4$ |
|  | 6.09 | 1 | 97 | 0.83 | 61.0 | 57.1 | 4.0–5.0 | $H_2SO_4$ |
|  | 6.40 | 1 | 92 | 1.09 | 59.6 | 56.5 | 4.0–5.0 | $H_2SO_4$ |
|  | 6.15 | 10 | 66 | 1.15 | 66.6 | 63.6 | 4.0–5.0 | $H_2SO_4$ |
|  | 6.03 | 1 | 76 | 1.10 | 60.4 | 57.9 | 4.8 | Itaconic acid |
|  | 6.03 | 10 | 66 | 1.46 | 65.6 | 62.4 | 5.0 | Itaconic acid |
|  | 6.15 | 10 | 74 | 1.15 | 66.9 | 64.0 | 4.9 | Itaconic acid |
|  | 6.00 | 10 | 66 | 1.57 | 66.0 | 61.1 | 2.2 | Itaconic acid |
|  | 5.64 | 10 | 87 | 1.35 | 60.2 | 60.1 | 2.8 | Itaconic acid |

F. Pfeifer, G. E. N. Nelson, C. Vojnovich, and L. B. Lockwood, U.S. Patent
73, Oct. 27, 1953.
illiliters of $N/10$ NaOH per hour for 10-ml. sample.

**duction in Surface Culture.** Information concerning the produc-
f itaconic acid by surface-culture methods has been presented by
hita (1929, 1931, 1937); Calam, Oxford, and Raistrick (1939);
vood and Reeves (1945); Moyer and Coghill (1945); Lockwood
Ward (1945); Lockwood and Moyer (1949); Vincenty et al. (1950);
ler Westhuizen et al. (1951); and others. Some data pertinent to
rmentation are presented in Table 167. The subject has been
ed in detail by Prescott and Dunn (1949). In view of the marked
ority of the submerged-culture process for making itaconic acid,
reference to the surface-culture method will not be made here.
terested reader is referred to the literature cited for details.

**very of the Acid.** After the fermentation is completed, the mold
um is separated from the spent liquor (beer) by filtration. Pfeifer
1952) used a recessed-plate filter for this purpose and found that
ids were unnecessary. The filtered liquor is reduced to 1/10 to 1/13
original volume by vacuum concentration. The hot, concentrated
allowed to crystallize in a special jacketed kettle equipped with a
le-speed agitator and facilities for controlling the temperature
g with water at 60°F. is satisfactory). The crystals of itaconic
re separated from the concentrated liquor by centrifuging in a
type centrifuge (Tolhurst). The crystals thus obtained are
with a minimum quantity of cold water and then scraped from
trifuge basket and dried in a vacuum tray drier under a vacuum
. and at a temperature of 125°F.

nother liquor from the centrifuge is evaporated to about one-half
me, and a second crop of crystals is obtained, which is returned
ixed with the filtered beer from a succeeding fermentation. The
liquor from the second crop of crystals is discarded. In the

manner thus outlined, Pfeifer and his associates were able t 90 per cent of the total acid as light tan crystals of 96 to 97 purity. In order to produce crystals of 99 per cent or greater pu necessary to dissolve the tan-colored crystals in hot water to yi centrated solution and add 2 per cent of carbon (based on the the crystals). The mixture is agitated for 30 min.; then an e amount of filter aid is added, and filtration follows, in which jacketed Sparkler filter is used. The solution is crystallized, cer and dried.

**Determination of the Acid in Fermentation Liquors.** In t mination of the itaconic acid content of a fermentation liquor, an may be obtained by titrating an aliquot sample with a standa solution, using phenolphthalein as the indicator. In order that th ing figure might be considered absolutely veracious, it would ha assumed that itaconic acid was the only acid produced by the mo this is not necessarily the case has been shown by Calam, Oxf Raistrick, who reported that several strains of *A. terreus* form oxalic, and succinic acids. Hence, unless the amounts of these ac duced by the mold are known, titration gives an estimate only.

Friedkin has developed a method (a modification of the Koppe method), for the direct determination of itaconic acid in ferm liquors. This involves the measurement of bromine absorption by acid in acid-buffered bromine water and depends on the fact that bromine at a pH of 1.2 reacts equimolecularly with itaconic acid b not react with glucose. The method is as follows:

One or 2 ml. (usually 2 ml.) of a uniform sample of the ferme liquor is introduced into a 125-ml. iodine flask by means of a To the sample is added 50 ml. of acid-buffered bromine water (at p The stopper of the iodine flask is then sealed with water to prev loss of bromine vapor. The flask is allowed to stand at room temp for 10 min. and is then placed in an ice bath. After cooling for 5 ml. of strong potassium iodide solution (made by dissolving 5 potassium iodide in 100 ml. of distilled water) is placed in the we rounding the stopper of the iodine flask. The stopper is carefully and the potassium iodide solution is sucked into the flask as a result partial vacuum created by cooling the flask plus its contents.

After allowing 10 min. for the reaction, the iodine released fr potassium iodide is titrated with 0.1 $N$ sodium thiosulfate, using as the indicator.

A blank containing 50 ml. of bromine water is treated in the manner as the sample of fermentation liquor.

The number of milliliters of 0.1 $N$ sodium thiosulfate required to with the iodine released from the potassium iodide by the 50-ml. of bromine water ($X$) minus the number of ml. of 0.1 $N$ sodium sulfate required to react with the iodine released by the bromine th not react with itaconic acid ($Y$) is equal to the number of millilit 0.1 $N$ itaconic acid. Therefore, $(X - Y)0.1$ = the number of millie

---

[1] W. F. Koppeschaar, *Zeit. anal. Chem.*, **15**: 233 (1876).

s of itaconic acid, and $(X - Y)0.0065$ = the weight in grams of the nic acid in the sample analyzed.

e reagent (bromine water) used in the analysis contains the follow- onstituents:

| | |
|---|---|
| Bromine | 1.0 ml. |
| Potassium bromide | 3.0 g. |
| Potassium chloride | 1.87 g. |
| 1.0 $N$ HCl | 48.5 ml. |
| Water (distilled) to | 500 ml. |

e bromine and potassium bromide are dissolved in a small portion stilled water, and the other ingredients are then added. The resultant s 1.2 ± 0.1, and hence no further adjustment is necessary. The nt should be stored in a dark bottle, preferably in a refrigerator.

TABLE 169. SOME APPLICATIONS OF ITACONIC ACID AND ITS DERIVATIVES

| Application | Reference |
|---|---|
| ating resins | Ellis, C.: U.S. Patent 2,195,362, Mar. 26, 1940.<br>D'Alelio, G. F.: U.S. Patent 2,288,315, June 30, 1940.<br>———: U.S. Patent 2,323,706, July 6, 1943. |
| esins | D'Alelio, G. F.: U.S. Patent 2,279,883, Apr. 14, 1942. |
| products (colorless) | D'Alelio, G. F.: U.S. Patent 2,279,883, Apr. 14, 1942.<br>———: U.S. Patent 2,279,881, Apr. 14, 1942.<br>———: U.S. Patent 2,279,882, Apr. 14, 1942.<br>———: U.S. Patent 2,279,885, Apr. 14, 1942.<br>———: U.S. Patent 2,298,039, Oct. 6, 1942.<br>———: U.S. Patent 2,310,731, Feb. 9, 1943. |
| ting-oil additives | Lippincott, S. B., and L. A. Mikeska: U.S. Patent 2,542,542, Feb. 20, 1951.<br>Giammaria, J. J.: U.S. Patent 2,616,849, Nov. 4, 1952.<br>———: U.S. Patent 2,616,853, Nov. 4, 1952.<br>Tutwiler, T. S.: U.S. Patent 2,637,698, May 5, 1953. |
| ange resins | D'Alelio, G. F.: U.S. Patent 2,340,110, Jan. 25, 1944.<br>———: U.S. Patent 2,340,111, Jan. 25, 1944. |
| c fibers | D'Alelio, G. F.: U.S. Patent 2,366,495, Jan. 2, 1945.<br>———: U.S. Patent 2,531,408, Nov. 28, 1950.<br>———: U.S. Patent 2,531,410, Nov. 28, 1950.<br>———: U.S. Patent 2,583,325, Jan. 22, 1952.<br>———: U.S. Patent 2,583,326, Jan. 22, 1952.<br>———: U.S. Patent 2,687,400, Aug. 24, 1954. |
| esins | Feagin, R. C., and C. H. Prange: U.S. Patent 2,318,845, May 11, 1943. |
| ers | Dorough, G. L.: U.S. Patent 2,384,239, Sept. 4, 1945. |
| litioners | Hedrick, R. M., and D. T. Mowry: U.S. Patent 2,625,529, 1953.<br>——— and ———: U.S. Patent 2,625,471, 1953. |
| rs | D'Alelio, G. F.: U.S. Patent 2,297,290, Sept. 29, 1942.<br>———: U.S. Patent 2,340,108, Jan. 25, 1944. |

According to Friedkin, the following substances, which may b
fermentation liquors, do not interfere with the analysis withi
mental accuracy: 15 per cent glucose, 1 *N* D-gluconolactone, 1
acid, 1 *N* aconitic acid, 1 *N* citric acid, saturated fumaric acid, 1
acid, 1 *N* malic acid, 1 *N* oxalic acid, 1 *N* succinic acid, and 1
acid.

**Applications.** Table 169 shows some of the applications of
acid. In addition, many compounds may be prepared from i
esters.

### References on Itaconic Acid

Calam, C. T., A. E. Oxford, and H. Raistrick: The Biochemistry of Micro
LXIII. Itaconic Acid, a Metabolic Product of a Strain of *Aspergill*
Thom, *Biochem. Jour.,* **33**: 1488–1495 (1939).

Friedkin, M.: Determination of Itaconic Acid in Fermentation Liquors,
*Chem., Anal. Ed.,* **17**: 637–638 (1945).

Kane, J. H., A. C. Finlay, and P. F. Amann: Production of Itaconic Acid, U
2,385,283, Sept. 18, 1945.

Kinoshita, K.: Formation of Itaconic Acid and Mannitol by a New Fil
Fungus, *Jour. Chem. Soc. Japan,* **50**: 583–593 (1929).

———: Über die produktion von Itaconsäure und Mannit durch einen neue
melpilze, *Aspergillus itaconicus, Acta Phytochim. Japan,* **5**: 271–287 (19

———: Physiological and Biochemical Studies of *Aspergillus itaconicus, Act*
*chim. Japan,* **9**: 159–187 (1937).

Larsen, H., and H. Hovden: Itaconic Acid by Fermentation, Norwegian
87,616, July 2, 1956.

Lockwood, L. B., and G. E. N. Nelson: Some Factors Affecting the Produ
Itaconic Acid by *Aspergillus terreus* in Agitated Cultures, *Arch. Biochem*
365–374 (1946).

———, K. B. Raper, A. J. Moyer, and R. D. Coghill: The Production and
terization of Ultraviolet-induced Mutations in *Aspergillus terreus.* I
chemical Characteristics of the Mutations, *Am. Jour. Botany,* 32(4):
(1945).

——— and M. D. Reeves: Some Factors Affecting the Production of Itaconic
*Aspergillus terreus, Arch. Biochem.,* **6**: 455–469 (1945).

——— and G. E. Ward: Fermentation Process for Itaconic Acid, *Ind. Eng.*
**37**: 405–406 (1945).

Moyer, A. J., and R. D. Coghill: The Laboratory-scale Production of Itaco
by *Aspergillus terreus, Arch. Biochem.,* **7**: 167–183 (1945).

Pfeifer, V. F., C. Vojnovich, and E. N. Heger: Itaconic Acid by Fermentati
*Aspergillus terreus, Ind. Eng. Chem.,* **44**: 2775 (1952).

———, G. E. N. Nelson, C. Vojnovich, and L. B. Lockwood: U.S. Patent 2
Oct. 27, 1953.

Raper, K. B., R. D. Coghill, and A. Hollaender: The Production and Chara
tion of Ultraviolet-induced Mutations in *Aspergillus terreus.* II. Cultu
Morphological Characteristics of the Mutations, *Am. Jour. Botany,* 32(3
176 (1945).

Van der Westhuizen, G. C. A., C. J. P. Spruit, and H. H. Sephton: Itaconi
I. Production by *Aspergillus terreus* from Unrefined Cane Sugar, *Jour*
*Chem.,* **I**: 356–360 (1951).

Vincenty, C.: *El Crisol (Puerto Rico),* **2**(1): 97; (2): 6 (1948).

———, P. M. Paradís, L. E. Cancel, D. Alcalá and R. Fernandez García: *B*
*Quimicos Puerto Rico,* p. 24.

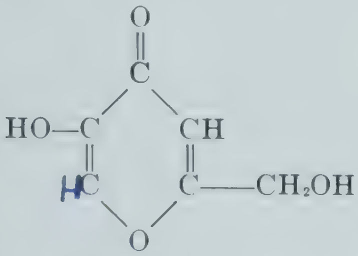
———, ———, ———, ———, and ———: *El Crisol (Puerto Rico),* **4**(4):
(1950).

## CHAPTER 31

# THE KOJIC ACID FERMENTATION

ic acid may be produced readily by microbiological means. At the t time it is produced commercially on a limited scale by mold tation.

ic acid (2-hydroxymethyl-5-hydroxy-gamma-pyrone) possesses the ing structural formula:



**erties.** Kojic acid is readily soluble in acetone and ethyl acetate; to the extent of 2.8 g. per 100 ml. of water at 20°C. and 6.8 g. per of water at 60°C.; slightly less soluble in ethanol than in water; htly soluble in chloroform, ether, and pyridine.

**rical.** Saito (1907) first isolated the acid as a by-product of the ation of steamed rice by *Aspergillus oryzae.* When ferric chloride led to an aqueous solution of the acid, an intense red color devel- Japanese biochemist, Yabuta, investigated the acid isolated and named it "kojic acid" (1912). The constitution of the acid blished by Yabuta in 1924.

ta-Mosca (1914) showed that kojic acid was produced by *A.* from sucrose, glucose, fructose, and glycerol. Wijkman (1924) l the acid by the fermentation of sucrose using an unnamed f *Aspergillus.* Three years later, Kinoshita (1927) indicated that of cobaltamines as sources of nitrogen caused increased yields of id from sucrose when using *A. oryzae.* When purpureo-cobaltic was added to a 10 per cent sucrose mash in a 0.5 per cent concen- a 33 per cent yield by weight of kojic acid was obtained in

27, Tamiya pointed out that several different aspergilli had the o produce kojic acid from sucrose. Among the molds mentioned *oryzae, A. flavus* var., *A. gymnosardae, A. awamori, A. candidus, tus, A. fumigatus,* and *A. giganteus.*

29. Challenger, Klein, and Walker published the results of which showed that kojic acid could be produced from xylose by

*A. oryzae*. During the same year Katagiri and Kitahara report formation of kojic acid from pentoses, gluconic acid, and other s

Since 1929, several papers have been published concernin aspects of the kojic acid fermentation. Titles to some of the nificant papers will be found in the reference list at the end of th

**Microorganisms Producing Kojic Acid.** Several molds of *Aspergillus* have the ability to produce kojic acid from suitabl containing nutrient solutions. In addition to the species enum the foregoing paragraphs, the following molds produce kojic *albus, A. effusus, A. nidulans, A. parasiticus, A. tamarii,* and *lium daleae*. From among the bacteria, several species of *Ac* may also form kojic acid under favorable conditions.

**Carbon Sources.** A fairly large number of carbon-contain stances have been fermented with the production of kojic acid by microorganisms. These substances include starches; dextrins charides, such as sucrose and maltose; the hexoses—glucose, mannose, and galactose; the pentoses—xylose and arabinose; ar tol, dulcitol, *i*-adonitol, inulin, inositol, glycerol, glycero-beta-ph dihydroxyacetone, gluconic acid, tartaric acid, and other substan best yields have, in general, been obtained from glucose and xylos

**Concentration of Carbon-containing Substance Used.** The trations of carbon-containing materials used have been vari approximately 5 to 30 per cent. May and his associates (193 sugar concentrations varying from 15 to 33 per cent in their work obtained highest yields with *A. flavus* when using a concentratio per cent glucose. Barham and Smits (1936) found a 15 per cent tration of xylose to be most suitable for fermentation. Katag Kitahara used 5 per cent concentrations of a large number of sub with satisfactory results in most cases. The same concentration h used by other workers.

**Nutrient Salts.** The types of nutrient salts used for kojic acid tion are illustrated in the paragraphs following.

May and his coworkers (1931) obtained satisfactory results whe the following salts in the concentrations recorded:

| | G./Liter of Glucose Solution |
|---|---|
| $MgSO_4 \cdot 7H_2O$ | 0.500 |
| KCl | 0.100 |
| $H_3PO_4$ | 0.054 |
| $NH_4NO_3$ | 1.125 |

The medium of Kinoshita (1927) often referred to as "medium K the following composition:

| | G. in 1.000 Ml. of Water |
|---|---|
| $MgSO_4 \cdot 7H_2O$ | 0.5 |
| $KH_2PO_4$ | 1.0 |
| $NH_4NO_3$ | 0.4 |

atagiri and Kitahara used media containing 0.01 per cent
$O_4 \cdot 7H_2O$, 0.1 per cent $KH_2PO_4$, 0.05 per cent $(NH_4)_2SO_4$, and 0.01
cent $CaCl_2$ with 5 per cent of carbon-containing substance.

ojic acid may be produced in the usual Czapek-Dox medium.

mmonium nitrate is very satisfactory as a source of nitrogen. Appar-
y better yields are obtained when the concentration of the salt is
l but sufficiently large to permit growth of the mold.

**H.** The optimum pH for the production of kojic acid under a given
f conditions must be determined by experimentation if this fact is
lready known. The range of pH 2 to 5 or above has been used by
us workers. Katagiri and Kitahara employed an initial pH of 5 to
the growth of *A. oryzae* but found that a pH of 2.4 stimulated
ation of kojic acid. A pH of 5.5 was found to be optimum for the
ntation of sucrose by Tamiya. Barham and Smits obtained highest
s when using a pH range of 2 to 3.5 in the fermentation of xylose
. *flavus*. These men advanced the opinion that the optimum pH for
ermentation was the lowest one that the organism would tolerate.
pare with the citric acid fermentation.)

e addition of calcium carbonate to a kojic acid fermentation results
reatly diminished yield of the acid. It is believed that the decreased
is due to a change in the pH of the mash.

**ect of Added Substances.** In a survey of 40 organic compounds,
and his associates found that ethylene chlorhydrin in a concentra-
f 100 mg. per liter produced a marked increase in the yield of kojic
n a period of 10 days.

lic, citric, formic, hydrochloric, and nitric acids inhibit the forma-
kojic acid by fermentation (Barham and Smits, 1934).

**perature.** The temperature range of 29 to 35°C. is optimum for
mentation. May and his associates advocated a temperature of
5°C. for *A. flavus* (a strain of the *A. flavus-oryzae* group, secured
hom as culture No. 3538). A temperature of 29 to 31°C. was used
ahara and Katagiri; one of 35°C., by Barham and Smits; and one
., by Gould. Sometimes the temperature may be dropped to 25°C.
r after the fermentation has proceeded at a higher temperature for
5 days, resulting in increased yields.

**ation of Fermentation.** The fermentation generally requires 9 to
s for completion, the period depending on the type of substrate,
of mold, temperature, pH, and other factors. After the sugar has
onsumed, the kojic acid may be utilized by the mold, resulting in
sed yields.

**ds.** Yields amounting to 50 to 60 per cent may be produced from
solutions by *A. flavus*. Table 170 illustrates the nature of the
btained from various compounds by Katagiri and Kitahara.

**tions.** One of the valuable characteristics of kojic acid is its
ty. Reactions may be effected at the hydroxyl groups, the nuclear
l group generally reacting first. Water-insoluble chelates, pyri-
yridones, esters, ethers, condensation products, halogen deriva-
d other products may be formed from kojic acid. Derivatives of

TABLE 170. AMOUNT OF KOJIC ACID PRODUCED BY *Aspergillus oryzae* *

(From 100 ml. of 20 days' culture containing 5 g. of substance)

| Substance | Kojic acid | | Observed by Sakaguchi (1932) | Ob T ( |
|---|---|---|---|---|
| | G. in 100 ml. | Yield on basis of sugar present, per cent | | |
| Glycogen | 0 | 0 | .. | |
| Inulin | 0.65 | 14.8 | .. | |
| Sucrose | 1.45 | 34.8 | + | |
| Maltose | 1.70 | 40.8 | .. | |
| Lactose | ± | ± | + | |
| Trehalose | 0.095 | 2.3 | .. | |
| Glucose | 0.75 | 18.1 | + | |
| Fructose | 0.40 | 10.2 | + | |
| Mannose | 0.23 | 5.8 | .. | |
| Galactose | 0.04 | 1.0 | + | |
| α-Methylglucoside | 0 | 0 | .. | |
| Rhamnose | 0 | 0 | + | |
| Arabinose | 0.08 | 2.0 | + | |
| Xylose | 0.40 | 10.2 | + | |
| Dihydroxyacetone † | 0.52 | 33.0 | + | |
| Glyceraldehyde | ± | ± | | |
| Methylglyoxal | 0 | 0 | | |
| Inositol ‡ | 0.04 | 0.9 | .. | + |
| Mannitol | ± | ± | + | + |
| Sorbitol ‡ | 0.4 | 10.2 | .. | + |
| Dulcitol ‡ | 0.4 | 10.2 | .. | + |
| Erythritol | ± | ± | + | + |
| Glycerol | 0.16 | 4.2 | + | ± |
| Na-glycerophosphate | + | + | | |
| Ethylene glycol | 0 | 0 | .. | 0 |
| Ethyl alcohol | 0 | 0 | + | |
| Methyl alcohol | 0 | 0 | ± | |
| Gluconic acid § | 0.34 | 9.4 | ± | 0 |
| Saccharic acid | 0 | 0 | .. | 0 |
| Lactobionic acid | ± | ± | | |
| Arabonic acid | 0 | 0 | | |
| Glyceric acid | 0 | 0 | | |
| Succinic acid | 0 | 0 | .. | ± |
| Tartaric acid | 0 | 0 | .. | 0 |
| Oxalic acid | 0 | 0 | | |
| Acetic acid | 0 | 0 | ± | 0 |
| Pyruvic acid | 0 | 0 | .. | 0 |
| Lactic acid | 0 | 0 | .. | 0 |
| Glycolic acid | 0 | 0 | .. | 0 |

---

* H. Katagiri and K. Kitahara, *Mem. Coll. Agr., Kyoto Univ.*, No. 26, *Chem.* 15, March, 1933.

† The mixture of 2 per cent dihydroxyacetone and 3 per cent α-methylglucosid used for the observation with dihydroxyacetone.

‡ Incubation for 14 days.

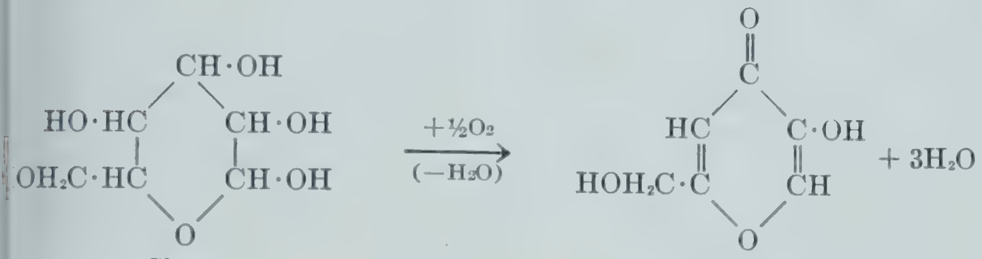
§ Incubation for 40 days.

acid, such as chlorokojic acid, may be used as intermediates for ormation of compounds which cannot be made directly from kojic

**ses.** Kojic acid has a number of potential uses. It might be used as nalytical reagent, particularly for iron; as an insecticide; as an anti- c; as an intermediate in the making of metal chelates; and for other oses.

**echanism of Kojic Acid Formation.** There have been several nes proposed to explain the production of kojic acid by fermentation. of these will be considered briefly.

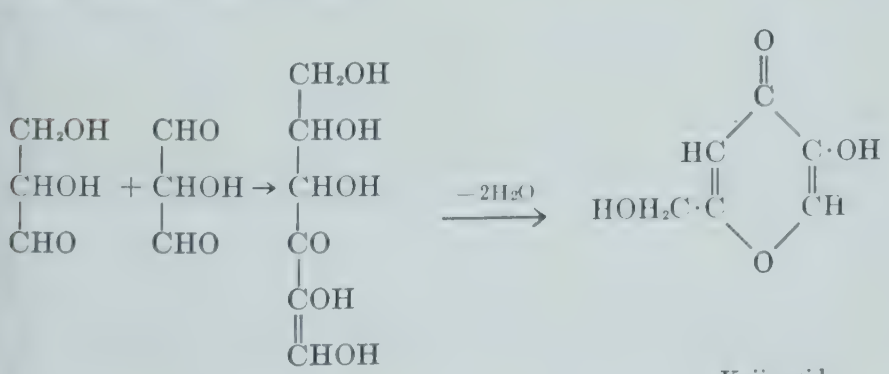
buta held that kojic acid arose directly from glucose by a simple ss of oxidation and dehydration:


Glucose

Kojic acid

oshita (1927) and Haworth (1929) offered a similar explanation e origin of kojic acid. The basis for this theory appears to be that e yields relatively large quantities of the acid in comparison with of the other carbon-containing materials. This theory does not 1 how kojic acid is formed from compounds containing less than on atoms, such as the pentoses, glycerol, and dihydroxyacetone, or -carbon compounds.

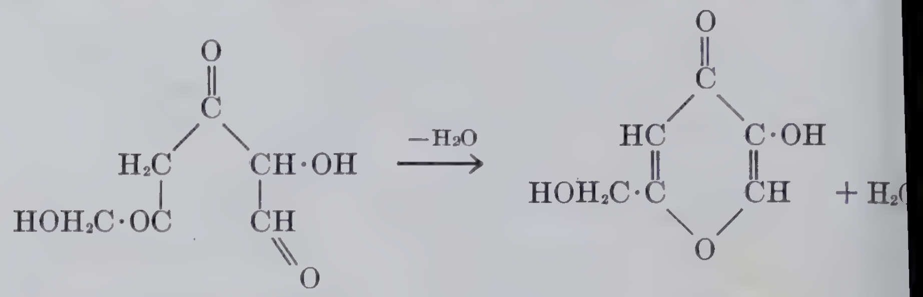
ellini and Gregorini (1930) advanced the suggestion that a pyrone s is synthesized from 3-carbon compounds. For example, 2 mole- f 3-carbon compounds may condense to form a molecule that by ation passes to kojic acid:


Kojic acid

s are readily formed chemically from acyclic polyketones through ation.

and his associates share the view advanced by Corbellini and ni that kojic acid is synthesized from some substance containing carbon atoms. They suggest that 1-hydroxyacetyl-3-formyl-3-

hydroxyacetone may be the precursor of kojic acid, although th compound has not been isolated from a kojic acid fermentation:



1-Hydroxyacetyl-3-formyl-3-hydroxyacetone

Kojic acid

Birkinshaw, Charles, Lilly, and Raistrick (1931) have propos theories to explain the mechanism of kojic acid formation. One suggests the formation of kojic acid through the condensation of ac hyde. This theory is based upon the fact that ethyl alcohol is freq found in mold fermentations, especially in those in which kojic one of the end products. The suggestion is also made that the pr of ethanol presupposes the occurrence of acetaldehyde as a pre Katagiri and Kitahara (1929) showed that acetaldehyde could be de by sulfite fixation in several different types of media ferment *A. oryzae* (Higuchi blue). Sakaguchi found that ethanol increas yield of kojic acid from glucose solutions. He therefore believe ethanol might be an intermediate in the fermentation.

The addition of fixing agents, such as sulfite and dimedon, to m being fermented by *A. tamarii* did not prevent kojic acid formatio could fixation products be isolated from the media, according to (1938).

Katagiri and Kitahara (1933) have reported that no kojic acid be detected in media that contained calcium hexosediphosphate o cium and magnesium hexosemonophosphates. Furthermore, these in gators were unable to obtain kojic acid from media containing acet hyde; acetone; or pyruvic, parapyruvic, or acetoacetic acids.

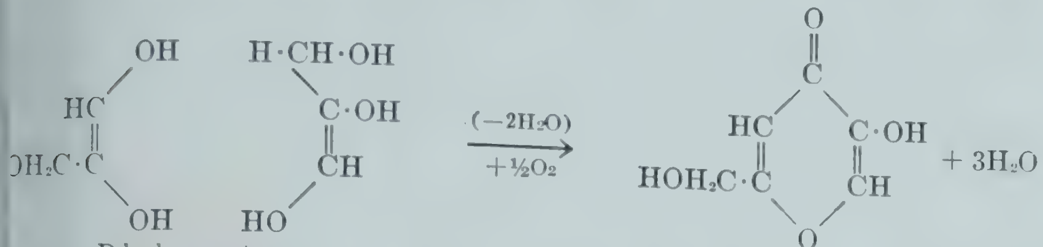
Thus, although acetaldehyde and ethanol are produced in some fermentations, there is no direct evidence to show that they are intern ate products in the formation of kojic acid.

The second theory proposed by Birkinshaw and his associates was a reserve carbohydrate would be anabolized from the carbon-conta substance in the medium and that subsequently the reserve carbohy would be hydrolyzed to a compound that could be converted to kojic

Research carried out by Gould has indicated that kojic acid is prob not produced from reserve carbohydrates. Gould grew mycelial ma *A. tamarii* on media containing several different carbon-containing stances. The mats were washed free of kojic acid, dried, and then gr to a fine powder. The powder was substituted for sugar in the Cza Dox medium. No kojic acid was produced in 90 days.

Tamiya proposed that from the various constituents of the me a hexose might be synthesized which would give rise to kojic acid.

In 1931, Challenger, Klein, and Walker reported that *A. oryzae* pro- ed a yield of more than 30 per cent of the theoretical from dihydroxy- tone. They proposed the following scheme:



Dihydroxyacetone

Kojic acid

llenger and his associates thus assumed that dihydroxyacetone or eraldehyde were the logical intermediate products in the formation ojic acid.

atagiri and Kitahara (1933) concluded, as the result of extensive arch, that dihydroxyacetone would be the most probable substance hose suggested to assume an important role in kojic acid formation. nstein and Bentley (1950; 1953*a*, *b*, *c*; 1956) have carried out exten- research concerning the mechanism of kojic acid formation by *A. ae* (IMI 17299) and *A. flavus-oryzae*, using compounds with labeled on atoms. As a result of their studies, during which they demon- ed (1953*b*) the presence of aldolase and triosephosphate isomerase in nolds, they concluded (1953*c*) that kojic acid biosynthesis may in- three different metabolic processes: (1) direct formation from glu- without splitting of the carbon chain, (2) formation of triosephos- s, and (3) the incorporation of small molecules, such as acetate, ate, and glycine, into carbon atoms 4, 5, and 6 of kojic acid. They ler, as a result of their work with $^{14}$C-labeled glucose and dihydroxy- ne (1953*a*), that the major pathway wherein kojic acid arises is by conversion of glucose. They believe (1956), based on studies with $^{4}$C] ribose as a substrate for *A. flavus*, that a C$_6$ precursor and kojic re formed from pentose via the transketolase and transaldolase ons. They postulate that the third pathway is an extension of the ing one and that the small molecules all form a common C$_3$ precur- hich is incorporated chiefly into carbon atoms 4, 5, and 6 of cid.

re 109 (page 616) presents two schemes of Arnstein and Bentley formation of kojic acid from pentoses by the transketolase and ldolase reactions.

### Some References concerning the Kojic Acid Fermentation

ous: Kojic Acid, Data Sheet 502, Chas. Pfizer & Co., Inc., New York, March, .

Chlorokojic Acid, Data Sheet 516, Chas. Pfizer & Co., Inc., New York, Feb- y, 1956.

Kojyl Palmitate, Data Sheet 517, Chas. Pfizer & Co., Inc., New York, Feb- y, 1956.

. H. R. V., and R. Bentley: Kojic Acid Biosynthesis from 1-C$^{14}$-Glucose, *ure*, **166**: 948 (1950).

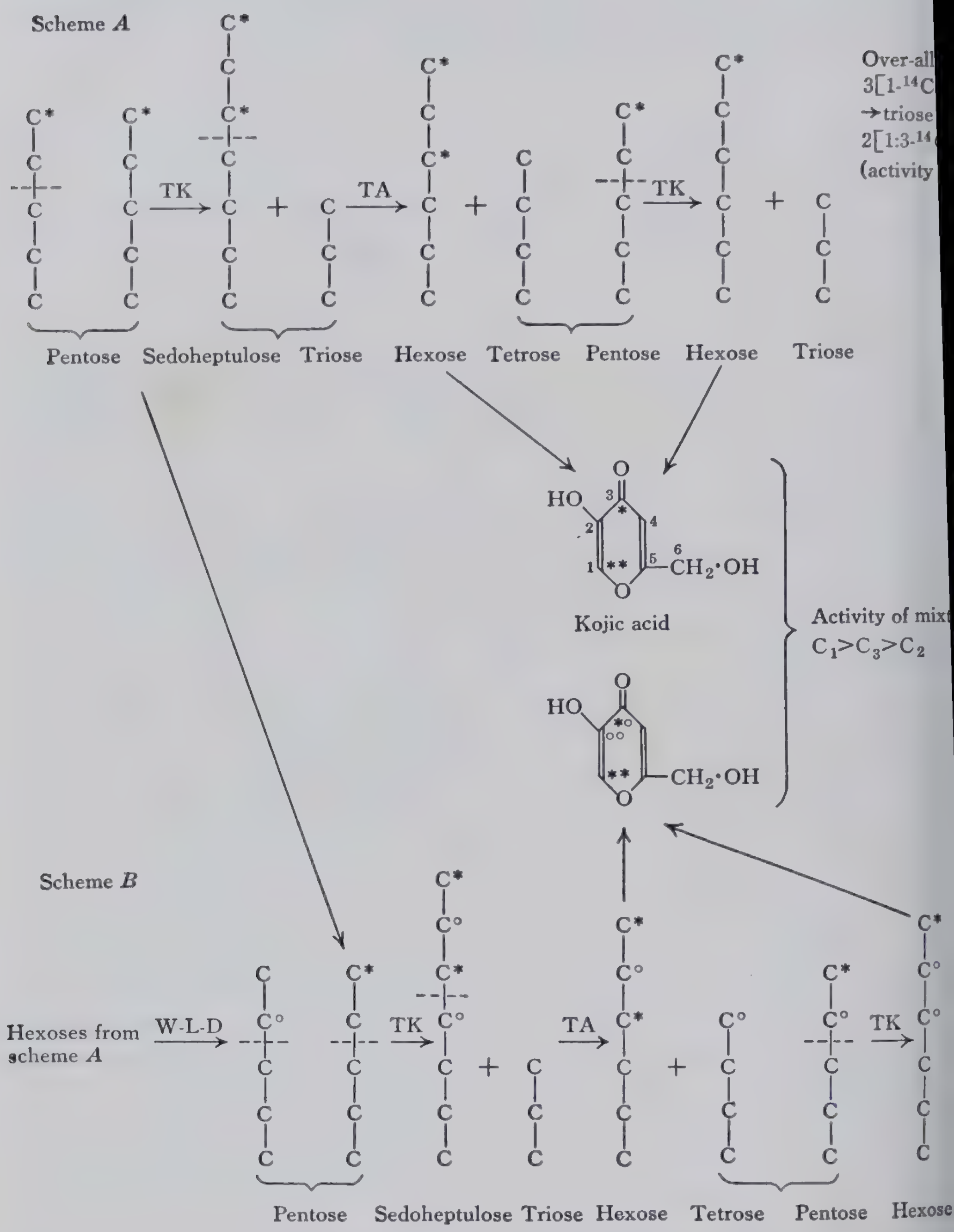

Scheme A
TK
TA
TK
Pentose
Sedoheptulose
Triose
Hexose
Tetrose
Pentose
Hexose
Triose
Over-all
3[1-14C
→triose
2[1:3-14
(activity
HO
O
3
2
4
1
5
6
CH2·OH
Kojic acid
Activity of mix
C1>C3>C2
Scheme B
Hexoses from scheme A
W-L-D
TK
TA
TK
Pentose
Sedoheptulose
Triose
Hexose
Tetrose
Pentose
Hexose

FIG. 109. Formation of $C_6$ precursor and kojic acid from labeled pentose vi ketolase and transaldolase reactions. [*Courtesy of H. R. V. Arnstein and R.* *Biochem. Jour.*, **62**: 403 (1956).

Abbreviations:

TK = transketolase reaction } reactants being shown by the symbol ⁓ and c
TA = transaldolase reaction } taking place at points indicated by dotted lin

W–L–D = Warburg–Lipman–Dickens oxidative mechanism. Asterisks and cir used to show the origin of labeled atoms, but have only semiquantitative signi Radioactivity in pentoses is differentiated as follows: C* original pentoses, C° formed from hexoses of Scheme *A* by the W–L–D mechanism. As a rough mation C° will therefore correspond to half the radioactivity indicated by C*

— and ———: The Biosynthesis of Kojic Acid. 1. Production from [1-$^{14}$C]- and [3:4-$^{14}$C$_2$]-Glucose and [2-$^{14}$C]-1:3-Dihydroxyacetone, *Biochem. Jour.*, **54**: 493–508 (1953*a*).

— and ———: 2. The Occurrence of Aldolase and Triosephosphate Isomerase in *Aspergillus* Species and Their Relationship to Kojic Acid Biosynthesis, *Biochem. Jour.*, **54**: 508–516 (1953*b*).

— and ———: 3. The Incorporation of Labelled Small Molecules into Kojic Acid, *Biochem. Jour.*, **54**: 517–522 (1953*c*).

— and ———: 4. Production from Pentoses and Methyl Pentoses, *Biochem. Jour.*, **62**: 403–411 (1956).

am, H. N.: Copper Precipitation Method for Kojic Acid Determination, *Ind. Eng. Chem.* (*Anal. Ed.*), **11**: 31 (1939).

– and B. L. Smits: Kojic Acid—A Review, *Trans. Kansas Acad. Sci.*, **37**: 91–113 1934).

– and ———: Production of Kojic Acid from Xylose by *Aspergillus flavus*, *Ind. Eng. Chem.*, **28**: 567–570 (1936).

nauer, K.: "Gärungschemische Praktikum," 2d ed., Springer-Verlag, Berlin, Vienna, 1939.

nshaw, J. H., J. H. V. Charles, C. H. Lilly, and H. Raistrick: Studies in the iochemistry of Micro-organisms. VII. Kojic Acid (5-Hydroxy-2-hydroxynethyl-γ-pyrone), *Trans. Roy. Soc. London*, **B220**: 127–138 (1931).

enger, F.: Some Fermentative Changes of Technical Interest, *Ind. Chemist*, : 97–100 (1930).

-, L. Klein, and T. K. Walker: The Production of Kojic Acid from Pentoses y *Aspergillus oryzae*, *Jour. Chem. Soc.*, July, 1929, pp. 1498–1505.

-, ———, and ———: The Formation of Kojic Acid from Sugars by *Aspergillus yzae*, *Jour. Chem. Soc.*, January, 1931, pp. 16–23.

llini, A., e B. Gregorini: La formazione dell'acido kojico dagli idrati di carbonio r azione dell' *Aspergillus flavus*, *Gazz. chim. ital.*, **60**: 244–256 (1930).

ua, A.: Azione del ferro sulla formazione di acido kojico per mezzo di *Aspergillus flavus*, *Gazz. chim. ital.*, **63**: 296–302 (1933).

nann, T. E.: Chemical and Physiological Properties of Kojic Acid, *Science*, **80**: (1934).

B. S.: The Metabolism of *Aspergillus tamarii* Kita. Kojic Acid Production, ochem. Jour., **32**: 797 (1938).

h, W. N.: "Constitution of Sugars," Longmans, Green & Co., Inc., New York, 9.

i, H., and K. Kitahara: The Formation of Kojic Acid by *Aspergillus oryzae*, *l. Agr. Chem. Soc. Japan*, **5**: 38–47 (1929).

and ———: The Formation of Kojic Acid by *Aspergillus Oryzae*, *Mem. Coll. ., Kyoto Univ.*, No. 26, *Chem. Ser.* 15, March, 1933, pp. 1–29.

ta, K.: Über die Ernährung der Pilze mit den Kobaltamminkomplexsalzen, *a Phytochim. Japan*, **3**: 31–50 (1927).

r, A. J., und L. H. C. Perquin: Über die Bedingungen der Kojisäurebildung ch *Aspergillus flavus* Link, *Biochem. Zeit.*, **266**: 82–85 (1933).

. K.: Die Überfuhrung von einfachen Zuckern in γ-Pyron-Derivate und die rstellung weiterer ungesattigter Anhydro-zucker, III, *Ber.*, **63B**: 25–34 (1934).

. E., A. J. Moyer, P. A. Wells, and H. T. Herrick: The Production of Kojic d by *Aspergillus flavus*, *Jour. Am. Chem. Soc.*, **53**: 774–782 (1931).

G. E. Ward, and H. T. Herrick: The Effect of Organic Stimulants upon the luction of Kojic Acid by *Aspergillus flavus*, *Zentr. Bakt. Parasitenk.*, Abt. II, 129–134 (1932).

. J.: Phosphorus Derivatives of Kojic Acid and Process of Preparation, U.S. ent 2,778,767, Jan. 22, 1957.

.: Über die Säurebildung bei *Aspergillus Oryzae* (Vorläufige, Mitteilung), *m. Mag. Tokyo*, **21**: 240 (1907).

i, K.: *Jour. Agr. Chem. Soc. Japan*, **8**: 265 (1932).

H.: Studien über die Stoffwechselphysiologie von *Aspergillus niger*, I, *Acta ochim. Japan*, **3**: 51–173 (1927).

———: Über die Verwendbarkeit von verschiedenen Kohlenstoffverbindun Bau- und Betriebstoffwechsel der Schimmelpilze, *Acta Phytochim. Ja* 1–129 (1932).

——— und T. Hida: Vergleichende Studien über die Säurebildung, die Atmu Oxydasereaktion und das Dehydrierungsvermögen von Aspergillusarten *Phytochim. Japan,* **4**: 343 (1929).

Traetta-Mosca, F.: La fermentazione di alcuni zuccheri, mediante l'*aspergillu cus,* con alcune considerazioni sulla fermentazioni alcoolica, *Ann. chim. a* 477–492 (1914).

——— e M. Preti: Azione dell' *Aspergillus Glaucus* sulla glicerina, *Gazz. chi* **51**: (II): 269–277 (1921).

Wijkman, N.: *Zeit. physiol. Chem.,* **132**: 104–108 (1924).

Yabuta, T.: On Kojic Acid, a new organic acid formed by *Aspergillus oryzae Coll. Agr., Tokyo Imp. Univ.,* **5**: 51–58 (1912).

———: Original Communication of the Eighth International Congress of A Chemistry (Appendix), **25**: 455–462 (1912).

———: *Jour. Chem. Soc. Japan,* **37**: 1185–1234 (1916).

———: The Constitution of Kojic Acid, a $\gamma$-Pyrone Derivative Formed by *gillus Oryzae* from Carbohydrates, *Jour. Chem. Soc. Japan,* **125**: 575–587 (

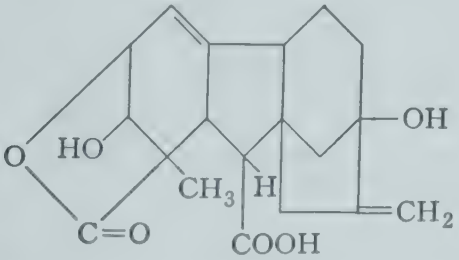
## CHAPTER 32

# GIBBERELLIC ACID AND THE GIBBERELLINS

ıe gibberellins, including gibberellic acid, are closely related sub- ces produced by *Gibberella fujikuroi* (Sawada) Wollenweber (the lial stage of *Fusarium moniliforme* Sheld.), a fungus which causes a ıse of rice seedlings, sometimes referred to as "foolish-seedling dis- " These substances, though differing somewhat, possess growth- ıoting properties which have produced rather amazing results. A study evaluation of the growth effects attributed to the gibberellins is ıpying the activities of many laboratories throughout the world.

ıe gibberellins include compounds designated as gibberellin $A_1$ ıiki, 1950) and gibberellin A (Stodola et al., 1955), which possess the ula $C_{19}H_{24}O_6$; gibberellin $A_2$ (Sumiki, 1950), which possesses the ula $C_{19}H_{26}O_6$; and gibberellin $A_3$ (Sumiki, 1950), gibberellin X lola et al., 1955), and gibberellic acid (Curtis and Cross, 1954), ı possess the formula $C_{19}H_{22}O_6$. Gibberellic acid and gibberellin X lentical. This substance is used chiefly for plant research.

: chemistry of gibberellic acid has been studied by Cross (1954), ɔlland and Ward (1954, 1956), and others. Gibberellic acid is a yclic dihydroxy-lactonic acid, according to Cross, to which the fol- ɡ structure has been tentatively assigned:



berellic acid is readily soluble in ethanol, methanol, acetone, sodium onate solution, and sodium acetate solution. It is only slightly · in water and ether.

**:orical.** Various aspects of this subject have been discussed by et al. (1954), Borrow et al. (1955), Stodola et al. (1955), Stodola 1958), and others.

ısawa (1926), a Formosan, showed that the symptoms of the *ıe* disease of rice seedlings could be produced by applying sterile, ·e filtrates of culture media of the fungus to healthy rice seedlings. ·dings were confirmed and extended by Seto (1928, 1932), Ito and

Shimada (1931), and other Japanese researchers, many from the
sity of Tokyo. Yabuta and Sumiki (1938) reported on the iso
gibberellin. Research papers concerned with the biochemistry a
aspects of gibberellin have been published by Yabuta, Hayash
Kitamura, and others.

Research and interest in the gibberellins was announced by
and Angel, of the U.S. Army Chemical Corps Biological Laborat
1950 (Stodola, 1956). As an outgrowth of this interest, Stodol
(1955) developed methods for producing and purifying gibber
and X.

In 1954, Curtis and Cross, of Herts, England, announced the
tion of gibberellic acid from the Raulin-Thom medium, using su
the carbon source. This report was followed by publications b
(1954), Mulholland and Ward (1954, 1956), Brian et al. (1954),
et al. (1955), and others.

Numerous papers concerned with effects of gibberellic acid a
gibberellins on plant growth have followed.

**Production.** The production of gibberellins and gibberellic a
been described by a number of scientists.

The first reports on the production of the gibberellins were pu
by the Japanese. Yabuta et al. (1939, 1940) discussed the cultur
ditions for producing gibberellin. Kitamura et al. (1953) describ
production of gibberellin by a submerged-culture method.

*Method of Stodola and Associates.* Stodola and coworkers des
the production of gibberellins A and X (gibberellic acid).

CULTURE. The mold used was *F. moniliforme* NRRL 2284. The
ulum for the aerated bottles was prepared by growing the mo
1 week on three long slants of potato dextrose agar at a temperat
24°C. Test tubes measuring 25 by 150 mm. were used for cul
the mold.

The medium was prepared as follows: Peeled and sliced pot
weighing 200 g., were boiled for 5 min. in 500 ml. of water. The res
infusion was filtered through cheesecloth, and the filtrate was resto
a volume of 500 ml. To this infusion were added (1) 100 ml. of
which contained 20 g. of dextrose, 0.2 g. of $CaCO_3$, and 0.2 g. of M
and (2) 400 ml. of water in which 15 g. of agar had been dissolved
medium was autoclaved for 20 min. at 15 p.s.i.g. before use.

INOCULUM IN AERATED BOTTLES. Four liters of the following m
were placed in a 9-liter bottle, equipped for aeration:

| | |
|---|---|
| Glucose | 2% |
| $NH_4Cl$ | 0.3% |
| $KH_2PO_4$ | 0.3% |
| $MgSO_4 \cdot 7H_2O$ | 0.3% |
| pH | Not adjusted |
| Octadecanol | 1 g. (added to bottle before sterilization) |

The mycelium from three long slants was removed and suspend
10 ml. of a 1:10,000 solution of sodium lauryl sulfonate in distilled

suspension was used to seed the 4 liters of medium in the 9-liter le. The mold was incubated, with aeration, for 75 hr. at 28°C. and l to inoculate the seed tank.

OCULUM IN SEED TANK. Twenty gal. of a medium of the same compo- n as that used in the aerated bottles were sterilized for 30 min. at C., cooled to 25°C. while a positive pressure of sterile air was kept in 30-gal. tank, and inoculated with 4 liters of seed material from a e. During growth of the mold, the temperature was held at 25°C., vas supplied at the rate of 0.25 volume per volume of medium per ite, and the medium was agitated at 150 r.p.m. When 30 hr. old, the re was used to inoculate the main fermentation medium.

RMENTATION. The medium used for the main fermentation, 154 gal., of the same composition as that used for the inocula, except that the se concentration was reduced to 1.25 or 1.5 per cent. It was sterilized nuously at 135°C. for 3 min. and possessed a poststerilization pH of ) 6.0. It was cooled to 25°C. and inoculated with 6 gal. of culture the seed tank. Subsequently, it was agitated at 100 r.p.m. and ed with 0.25 volume of air per volume of medium per minute. The ation of foam was automatically controlled through the use of er cent of octadecanol in 95 per cent of alcohol. The fermentation omplete in about 65 hr., at which time the glucose concentration ecreased to 0.15 per cent or lower.

LDS. From 160 gal. of the fermented medium, Stodola and associ- vere able to recover about 12 g. of gibberellin of crude crystalline sition and about 15.5 g. of crystalline fusarinic acid. Gibberellin X e formula $C_{19}H_{22}O_6$.

*hods of Borrow and Associates.* Borrow and his coworkers (1955) l the production of gibberellic acid by surface- and submerged- methods, using *G. fujikuroi* (strain 917). Yields of 40 mg. per ere obtained in 14 days at 25°C. by use of the Raulin-Thom n containing 2.5 per cent sucrose and adjusted to an initial pH of 5. approaching 200 mg. per liter were secured by the submerged- method. The fungus was cultured in 80-liter batches in a stainless- rmentor at 25°C. The Raulin-Thom medium containing 4 per cent , and usually at an initial pH of 5.5, was used. (An initial pH of 3.5 to 5.5 appeared to be satisfactory for gibberellic acid produc- Sterile air was supplied at the rate of 0.3 to 0.5 volume per volume ium per minute. Yields increased steadily up to about 18 days.

rding to Borrow and coworkers, gibberellic acid production may uring the phase of active mycelial growth. However, 75 per cent robably more) of the acid accumulated after the maximum dry of mycelium had been attained. During this time, the rapid of sugar and ammonia-nitrogen had stopped.

**ods of Application to Plants.** Gibberellic acid, or the gibberel- y be applied to any part of the plant. As a lanolin paste, it may ed in concentrations of up to 1 per cent to the stems, petioles, or In aqueous solution, it may be sprayed onto the leaves, petioles, fruits, or other parts; used as a dip for tubers; used as a soak for corms, bulbs, roots, or seeds; or placed in small droplets on gro

The usual concentration of gibberellic acid in the spray, di
may vary from as low as 0.001 p.p.m. to as high as 1,000 p.p.m.

When the product is to be used in aqueous solution, a measure
is dissolved in a few milliliters (4 to 5) of 95 per cent ethanol;
agent, such as Tween 20, is added; and the solution is brou
specific volume with water. By using this as a stock solution, t
sary concentrations may be prepared.

A 1 per cent lanolin paste is prepared as follows: 12.5 mg. of g
acid is added to 250 mg. of Tween 20, warmed gently, and mi
then 1 g. of melted lanolin is added. The ingredients are uniform

**Effects.** One of the pronounced effects of gibberellic acid, or
berellins, is to cause a rapid lengthening of the stems and inter
plants (Yabuta and Hayasi, 1939; Yabuta et al., 1941c, 1943; Bri
1954; Brian and Hemming, 1955; Marth et al., 1956; Rappapo
Kato, 1956; Lang, 1956; Bukovac and Wittwer, 1956; Witt
Bukovac, 1957; and others). Brian and associates (1955) have at
this effect to increased cell growth and not to increased cell divisi

The leaves of treated plants may be lighter green in color tha
of untreated plants. Likewise, rapid and prolonged stem elongati
be associated with decreased root growth (Marth et al., 1956).

Pea seedlings grown in nutrient water solution developed elongat
internodes but leaves of normal size. Wheat plants grown under
conditions showed increased length of stem internodes and lea
also narrower and paler leaves. The total weights of the peas an
were increased, the increase being due chiefly to carbon (Brian
1954).

The leaf petioles of celery increased in thickness and doubled in
within 2 or 3 weeks when grown in soil cultures in the greenhouse
vac and Wittwer, 1956). The fresh and dried weights of the
increased up to 50 per cent.

Gibberellins, when sprayed on tomato fruit clusters in concent
of 2 to 1,000 p.p.m., are effective in setting the fruit in the abs
pollination (Wittwer and Bukovac, 1957). Flowering is hasten
fruits are often seedless.

Gibberellic acid breaks up the dormancy of potatoes (Rap
1956). One lot of russet Burbank potatoes was dipped for 1.5
25 p.p.m. of gibberellic acid; another lot was dipped in water.
per cent of the potatoes treated with gibberellic acid sprouted in 1
whereas only 11 per cent of the untreated ones sprouted.

The development of flowers in plants may be hastened in some ins
and delayed in others (Marth et al., 1956). The flowers of dahlia
geraniums, petunias, and certain other plants open earlier (Wittw
Bukovac, 1957). Effects of gibberellin on other flower crops hav
described by Kohl and Kofranek (1957).

The soaking of seeds in gibberellic acid has accelerated their ger
tion and the emergence of seedlings in peas, beans, and certain
plants (Wittwer and Bukovac, 1957).

lower formation and seed production in carrots and other plants has n hastened by the use of gibberellic acid.

he new growth of young forest trees, such as the maple, tulip poplar, willow oak, is much increased through the use of gibberellic acid; ever, with similar treatment two species of pine and white spruce onstrated only a slight amount of increase in growth of new shoots arth et al., 1956).

ibberellin stimulated the germination of barley, rice, and wheat grains increased the amount of amylase in germinated barley and wheat ns (Hayasi, 1940*b*). However, it did not activate the amylase of solution. It had no effect on the growth, fermentation, and size of t cells (Hayasi, 1940*a*).

e activities of peroxidase and invertase in the seventh leaf sheaths ce plants cultivated in nutrient solution containing gibberellin were ased markedly. However, the activities of phosphatase, acetyl- ase, maltase, $\beta$-glucosidase, $\alpha$-galactosidase, $\beta$-galactosidase, amylase, e, dipeptidase, ascorbic acid oxidase, and catalase were decreased yashi, Murakami, and Matsunaka, 1956).

r further details on this fascinating subject, the reader is referred to asic research reports.

**plications.** Although gibberellic acid and gibberellins are being ifactured by a number of commercial concerns on a relatively small the cost of these compounds is relatively high. As Wittwer and vac (1957) have indicated, their use will be restricted for a while ps of high value which may be raised in the greenhouse, nursery, or n. Their use in the setting of fruit and seed production appears to be ally promising. It has been suggested that they may stimulate the of plants through stages in which they are particularly susceptible ase, accelerate the growth of slow-growing plants, increase the dry of forage crops at harvest, and hasten the seed production of bles, such as beets and cabbages, which are biennial crops.

### Some References concerning Gibberellic Acid and the Gibberellins

ous: Amazing Gibberellic Acid, *Agr. Research*, **5**(3): 12 (1956).

Gibberellins for Growth, *Chem. Eng. News*, September, 1956, pp. 4496, 4501.

"Gibrel," Merck & Co., Inc., Rahway, N.J. (Contains information regarding use of gibberellic acid and 188 abstracts.)

A., P. W. Brian, V. E. Chester, P. J. Curtis, H. G. Hemming, C. Henehan, G. Jeffreys, P. B. Lloyd, I. S. Nixon, G. L. F. Norris, and M. Radley: Gib- llic Acid, a Metabolic Product of the Fungus *Gibberella fugikuroi*: Some ervations on Its Production and Isolation, *Jour. Sci. Food Agr.*, **6**: 340–348 5).

. W., G. W. Elson, H. G. Hemming, and M. Radley: The Plant-growth- oting Properties of Gibberellic Acid, a Metabolic Product of the Fungus *rella fugikuroi*, *Jour. Sci. Food Agr.*, **5**: 602–612 (1954).

d H. G. Hemming: The Effect of Gibberellic Acid on Shoot Growth of Pea lings, *Physiol. Plantarum*, **8**: 669–681 (1955*a*).

——, and M. Radley: A Physiological Comparison of Gibberellic Acid with Auxins, *Physiol. Plantarum*, **8**: 899–912 (1955*b*).

M. J., and S. H. Wittwer: Gibberellic Acid and Higher Plants, *Mich. State Agr. Quart. Bull.*, **39**(2): 307–313 (1956).

Cross, B. E.: Gibberellic Acid. Part I, *Jour. Chem. Soc.*, 1954, pp. 4670–467

Curtis, P. J., and B. E. Cross: Gibberellic Acid, a New Metabolite from t Filtrates of *Gibberella fugikuroi, Chemistry & Industry,* 1954, p. 1066.

Hayashi, T., Y. Murakami, and S. Matsunaka: Biochemical Studies on Fungus. Part XXXVI. The Physiological Action of Gibberellin. VIII in the Activities of Various Enzymes in Leaf-sheaths of Rice Plant with Gibberellin, *Bull. Agr. Chem. Soc. Japan,* **20**(4): 159–164 (1956).

Hayasi, T.: Biochemistry of "Bakanae" Fungus of Rice. V. Effect of Gib Growth, Fermentation and Size of Yeast Cells, *Jour. Agr. Chem. Soc. J* 386–388 (1940*a*).

———: VI. Effect of Gibberellin on Activity of Amylase in Germinat Grains, *Jour. Agr. Chem. Soc. Japan,* **16**: 531–538 (1940*b*).

Ito, S., and S. Shimada: *Ann. Phytopath. Soc. Japan,* **2**(4): 322–338 (1931).

Kato, J.: Effect of Gibberellin on Elongation, Water Uptake, and Resp Pea-stem Sections, *Science,* **123**: 1132 (1956).

Kitamura, H., A. Kawarada, Y. Seta, N. Takahashi, T. Otsuki, and Y. Sum Production of Gibberellin by Submerged Culture, *Jour. Agr. Chem. So* **27**: 545–549 (1953).

Kohl, H. C., Jr., and A. M. Kofranek: Gibberellin on Flower Crops, *Calif. Ag* 5 (1957).

Kurosawa, E.: Experimental Studies on the Nature of the Substance Exc "Bakanae" Fungus, *Trans. Nat. Hist. Soc. Formosa,* **16**: 213–227 (1926).

Marth, P. C., W. V. Audia, and J. W. Mitchell: Effects of Gibberellic Acid on and Development of Plants of Various Genera and Species, *Botan. Gaz.,* 106–111 (1956).

———, ———, and ———: Gibberellic Acid—A Plant Regulator, *U.S. De* *Mimeographed Rept.* HCRB-6, September, 1956.

Mulholland, T. P. C., and G. Ward: Gibberellic Acid. Part II. The Struct Synthesis of Gibberene, *Jour. Chem. Soc.,* 1954, pp. 4676–4681.

——— and ———: Gibberellic Acid. Part III. Synthesis of Fluorenone-4: boxylic Acid, *Jour. Chem. Soc.,* 1954, pp. 2415–2417.

Ohrmund, M., J. L. Rowe, and C. Leben: A Partial List of References on t berellins, Eli Lilly Co., Research Laboratories, Indianapolis, 1956.

Rappaport, L.: Growth Regulating Metabolites, *Calif. Agr.,* **10**(12): 4, 11 (19

Stodola, F. H.: The Isolation, Characterization and Chemical Properties of t berellins, paper presented at symposium on Natural Plant Growth Reg Other than Auxin, Storrs, Conn., Aug. 28, 1956.

———: Source Book on Gibberellin, 1828–1957, *U.S. Dept. Agr., ARS*-71-11,

———, K. B. Raper, D. I. Fennell, H. F. Conway, V. E. Sohns, G. T. Langf R. W. Jackson: The Microbiological Production of Gibberellins A and *Biochem. and Biophys.,* **54**: 240–245 (1955).

Sumiki, Y.: The Chemical Constitution of Plant Growth Hormones and Mec of Their Action, *Jour. Jap. Chem.,* **4**: 306–314 (1950).

Wittwer, S. H., and M. J. Bukovac: Gibberellins—New Chemicals for Crop tion, *Mich. State Coll. Agr., Quart. Bull.,* **39**(3): 3–28 (1957).

Yabuta, T., et al.: *Jour. Agr. Chem. Soc. Japan,* **14**: 1526 (1938); **15**: 257, 4 (1939); **16**: 1157 (1940); **17**: 527, 673, 721, 894, 975, 1001 (1941); **18**: 207 **19**: 244, 396 (1943); **20**: 52 (1944); **24**: 395 (1951); **25**: 159, 503 (1951–19

——— and T. Hayasi: Biochemistry of the "Bakanae" Fungus of Rice. II. I of Gibberellin, the Active Principle which Produces Slender Rice Seedling *Agr. Chem. Soc. Japan,* **15**: 257–266 (1939).

——— and Y. Sumiki: *Jour. Agr. Chem. Soc. Japan,* **15**: 1209–1220 (1939).

## CHAPTER 33

# THE FUMARIC ACID FERMENTATION

maric acid ($HOOC\cdot CH{=}CH\cdot COOH$), an unsaturated acid, is pro-
l by several molds, principally species of the genus *Rhizopus*. Other
a of the family Mucoraceae, namely, *Circinella*, *Cunninghamella*,
*Mucor* (Foster and Waksman, 1939), also have the ability to produce
ric acid from nutrient sugar solutions. At least one species of *Asper-*
and one of *Penicillium* have been credited with the production of
cid.

rlich (1911) first reported on the formation of fumaric acid by molds.
ere is much variation in acid production even in strains that appear
identical morphologically. For example, Foster and Waksman have
ed the case of acid production by races of a strain of *R. nigricans*.
emale race produced fumaric acid from a nutrient glucose solution,
the male race failed to produce fumaric acid under the same and a
y of conditions. These results were not characteristic of sexual pairs
eral.

narily, molds produce only small quantities or traces of the acid,
least one strain of *R. nigricans* has the ability to convert 40 to 50
it of the sugar consumed to fumaric acid.

r acids, for example, lactic acid, are frequently produced simul-
sly with fumaric acid (see Chap. 34).

iderable information concerning the fumaric acid fermentation
obtained through a study of the papers cited at the end of this
. Birkinshaw (1937), Lockwood and Moyer (1938), and Foster
reviewed some of the literature concerning the production of
acid by molds. Foster and Waksman carried out extensive re-
concerning fumaric acid production by the genus *Rhizopus*, with
reference to the effect of zinc on growth and acid production.
(1952) discussed the conditions concerning the formation of
acid by *Rhizopus* species.

ble 171, some data concerned with molds that produce fumaric
summarized.

**sman Process.** Waksman patented a process for producing
acid from various carbohydrates and carbohydrate-containing
s, such as monosaccharides, molasses, and starch, by the use of
fungi, particularly of the order Mucorales. Although strains of
of *Cunninghamella*, *Circinella*, and *Rhizopus* may be employed,
f *R. nigricans* are preferred.

TABLE 171. SOME MOLDS PRODUCING FUMARIC ACID

| Molds | Investigators | re<br>re |
|---|---|---|
| *Aspergillus fumaricus* | Wehmer(25,26) * | 191 |
| *A. fumaricus* | Thies(21) | |
| *Circinella* sp. | Foster and Waksman(10) | |
| *Cunninghamella* sp. | Foster and Waksman(10) | |
| *Mucor* sp. | Foster and Waksman(10) | |
| *M. stolonifer* (*R. nigricans*) | Ehrlich(6,7) | 191 |
| *M. stolonifer* (*R. nigricans*) | Butkewitsch and Federoff(3,4,5) | 192 |
| *M. stolonifer* (*R. nigricans*) | Gottschalk(12) | 1 |
| *Penicillium griseo-fulvum Dierckx* | Raistrick and Simonart(17) | 1 |
| *Rhizopus japonicus* | Takahashi, Sakaguchi, and Asai(20) | 192 |
| *R. japonicus* | Meder(16) | 1 |
| *R. nigricans* | Waksman(22) | 1 |
| *R. nigricans* | Kane, Finlay, and Amann(13) | 1 |
| *R. niveus* | Takahashi and Sakaguchi(19) | 1 |
| *R. oryzae* | Ward, Lockwood, May, and Herrick(24) | 1 |
| *R. oryzae* | Lockwood, Ward, and May(15) | 1 |
| *R. pseudochinensis* | Takahashi and Asai(18) | 19 |
| *R. shanghaiensis* | Takahashi and Sakaguchi(19) | 19 |
| *R. tritici* | Takahashi and Sakaguchi(19) | 19 |

* The figures in parentheses refer to the bibliography at the end of this chapte

The mold may be grown and the acid produced in a single medi but preferably the mold is grown in one stage in a special medium under conditions designed to encourage growth, and the acid is produ in a second stage through the use of one or more replacement media u conditions that stimulate acid production. Growth and acid produc may take place by stationary surface-culture methods or by submer culture methods.

The growth medium contains sources of carbohydrate, nitrogen, nu ent salts, and trace elements or catalytic agents. A 5 to 15 per cent c centration of carbohydrate may be employed. Ammonium sulfate, s of ammonia, and urea are satisfactory nitrogen sources, but ammon sulfate is preferred. A concentration of 0.2 per cent $(NH_4)_2SO_4$ is s able, although the concentration of nitrogen may range from 0.2 to 0. per liter of medium. Additional nutrient salts may be supplied as per cent of $MgSO_4 \cdot 7H_2O$ and 0.05 per cent of $K_2HPO_4$.

Certain elements or salts are used by Waksman to stimulate m growth or fumaric acid production. Zinc, as $ZnSO_4 \cdot 7H_2O$, is added to culture medium in concentrations of 1 to 10 mg. per liter of solut containing 50 to 150 g. of carbohydrate to encourage rapid formation the mycelium. In order that growth may be followed at once by maximum production of fumaric acid, the effect of the zinc is balan with a salt of iron, for example $(Fe)_2(SO_4)_3$, in a concentration of 1 to mg. per liter. Zinc and iron are associative in their effects when the ra zinc to iron is about 1:2 and also when the initial concentration arbohydrate is 20 to 30 per cent. Traces of manganese and copper be added to the culture medium to accentuate the effect of the

he medium is sterilized with heat and inoculated with spores, or a ension of germinated spores, of a selected strain of *R. nigricans.* The ed medium is incubated at 28 to 35°C. to encourage growth of the l. The latter is allowed to grow as a pellicle on the surface of the cul- medium, or it is grown submerged in the medium while being agitated ration or mechanically at atmospheric or increased air pressures. A e-like support is recommended for surface culture of the mold, which res 2 to 7 days. Submerged cultures require 24 to 60 hr. for growth. fumaric acid formed during the growth phase is neutralized with um carbonate or other alkali. At the completion of this phase, the h solution is removed from the mycelium by draining, siphoning, or ifuging and saved for fumaric acid recovery.

ring the second stage (replacement phase), a solution of carbo- te (up to 20 per cent in concentration), not containing supple- ary nitrogen or nutrient mineral salts, is added to the mold mycelium. ent calcium carbonate to neutralize the fumaric acid expected (up roximately 50 per cent of the carbohydrate) may be added, or the nay be neutralized periodically with KOH or NaOH to a pH of .5. Substances to stimulate fumaric acid formation (accelerators) lded to the replacement medium. For example, 0.1 to 1 g. each of $(SO_4)_3$ and of $MgSO_4 \cdot 7H_2O$ may be added to 1 liter of solutions ning up to 20 per cent of carbohydrate, or 0.3 to 1 g. each of $)_4$ and $MgSO_4 \cdot 7H_2O$ may be added to solutions containing about of carbohydrate per liter. The solutions are incubated at 28°C. for lays.

ision of a surface support for the mycelium in the case of station- face-culture fermentation and of aeration, agitation, and air under e in the case of submerged-culture fermentation accelerates the ion of fumaric acid.

replacement of the carbohydrate solution may be repeated until yme system of the mold is impaired.

**e-Finlay-Amann Process.** A process for producing fumaric acid, lt of this acid, with traces only of other acids has been patented ne, Finlay, and Amann (1943). The mold used is a strain of the *Mucor*, *Aspergillus*, or *Rhizopus*. A selected strain of *R. nigricans* cularly suitable.

ric acid is produced by submerged-culture methods in a medium ng a carbohydrate, nutrient salts, and a neutralizing agent. . fructose, invert sugar, sucrose, maltose, molasses, sirups, starches, materials may serve as the carbohydrate source. Nutrient salts supplied as potassium acid phosphate and ammonium sulfate. lium may be mechanically agitated, aerated, or both, at atmos- r elevated pressures. An oxygen-containing gas may be substi- air. One illustration of the process follows:

A 30.25-lb. amount of hydrated glucose and small quantities of
salts were diluted with water to make 50 gal. of solution. To th
of calcium carbonate was added. The mixture was sterilized by
cooled, and seeded with 3 liters of a selected culture of *R. nigrica*
fermentation medium was stirred at 40 r.p.m., while a stream of
passed through it. Incubation was at a temperature of 28 to 32
fermentation was stopped after 3 days and the liquor analyzed.
of 12.5 lb. of fumaric acid was obtained.

### References on Fumaric Acid

1. Bernhauer, K., und H. Thole: Über die Säurebildung durch Rhizo
I. Die Bildung von Apfelsäure bei der Fumarsäuregärung, *Bioche*
**287**: 167–171 (1936).
2. Birkinshaw, J. H.: Biochemistry of the Lower Fungi, *Biol. Rev.*, **12**: 357
3. Butkewitsch, W., und M. W. Federoff: Über Bildung von Fumarsäure
Zuckerculturen von *Mucor stolonifer* (*Rhizopus nigricans*) und sein
zur Brenztraubensäure, *Biochem. Zeit.*, **206**: 440–456 (1929).
4. ——— und ———: Über die Umwandlung der Essigsäure durch *Mucor st*
in Bernstein- und Fumarsäure und das Verfahren zur Trennung und
tativen Bestimmung dieser Säuren, *Biochem. Zeit.*, **207**: 302–318 (1929
5. ——— und ———: Über die Verhaltnisse zwischen Essig-, Bernstein-,
und Oxalsäure in den Kulturen von *Mucor stolonifer* und einigen
Pilzen, *Biochem. Zeit.*, **219**: 87–102 (1930).
6. Ehrlich, F.: Über die Bildung von Fumarsäure durch Schimmelpilze, *B*
3737–3742 (1911).
7. ———: Über Fumarsäure—Gärung des Zuckers, *Ber.*, **52**: 63–64 (1919).
8. Foster, J. W.: "Chemical Activities of Fungi," Academic Press, Inc., New
1949, pp. 351–377.
9. ——— and S. A. Waksman: Fumaric Acid Formation Associated with Se
in a Strain of *Rhizopus nigricans*, *Science*, **89**: 37 (1939).
10. ——— and ———: The Production of Fumaric Acid by Molds Belonging
Genus *Rhizopus*, *Jour. Am. Chem. Soc.*, **61**: 127 (1939).
11. ——— and ———: The Specific Effect of Zinc and Other Heavy Me
Growth and Fumaric-acid Production by *Rhizopus*, *Jour. Bact.*, **37**: 599
12. Gottschalk, A.: Biochemische Synthese von Fumarsäure aus Brenztraube
*Zeit. physiol. Chem.*, **152**: 136–143 (1926); **172**: 314 (1927); **182**: 311 (1
13. Kane, J. H., A. Finlay, and P. F. Amann: Production of Fumaric Aci
Patent 2,327,191, Aug. 17, 1943.
14. Lockwood, L. B., and A. J. Moyer: The Production of Chemicals by Filam
Fungi, *Botan. Rev.*, **4**: 140 (1938).
15. ———, G. E. Ward, and O. E. May: The Physiology of *Rhizopus oryzae*
*Agr. Research*, **53**: 849–857 (1936).
16. Meder, H.: Über die Bedingungen der Bildung von Fumarsäure als Endp
des Stoffwechsels bei Rhizopus-Arten, *Ber. schweiz. botan. Ges.*, **62**:
(1952).
17. Raistrick, H., and P. Simonart: XXIX 2:5-Dihydroxybenzoic Acid (G
Acid). A New Product of the Metabolism of Glucose by *Penicillium*
*fulvum*, Dierckx, *Biochem. Jour.*, **27**: 628–633 (1933).
18. Takahashi, T., and T. Asai: On the Products of Fermentation by Mucor
II. The Products of Fermentation in Presence of Ca-carbonate, *Zentr.*
*Parasitenk.*, Abt. II, **89**: 81–84 (1933).
19. ——— and K. Sakaguchi: Studies on the Acid Formed by *Rhizopus*
*Jour. Agr. Chem. Soc.-Japan*, **1**(5): 46 (1925).
20. ———, ———, and T. Asai: *Bull. Agr. Chem. Soc. Japan*, **2**: 5 (1926);
(1927).
21. Thies, W.: Untersuchungen über den Einfluss der Bedingungen auf die

bildung des Schimmelpilzes *Aspergillus fumaricus, Zentr. Bakt. Parasitenk.*, Abt. II, **82**: 321–347 (1930).

Waksman, S. A.: Process for the Production of Fumaric Acid, U.S. Patent 2,326,986, Aug. 17, 1943.

—— et J. W. Foster: Effet du zinc sur la végétation de *Rhizopus nigricans* et la production d'acide par cet organisme, *Compt. rend.*, **207**: 483 (1938).

Ward, G. E., L. B. Lockwood, O. E. May, and H. T. Herrick: Studies in the Genus *Rhizopus*. I. The Production of Dextrolactic Acid, *Jour. Am. Chem. Soc.*, **58**: 1286–1288 (1936).

Wehmer, C.: Über Fumarsäure—Gärung des Zuckers, *Ber.*, **51**: 1663–1668 (1918).

——: Abnahme des Säuregärungsvermögen und Anderung der Säure bei einem Pilz (Gluconsäure statt Fumarsäure—Gärung), *Biochem. Zeit.*, **197**: 418–432 (1928).

## CHAPTER 34

# THE PRODUCTION OF LACTIC ACID BY MOLDS

**Historical.** In 1894 the suggestion was made by Eijkmann th acid formed by *Mucor rouxii* was lactic acid. Chrzaszcz conf Eijkmann's suggestion in 1901.

Patents were issued to Boullanger (1899) for the production of acid by a mold described as "Lactomyces," an organism now believ be a species of *Rhizopus.*

Saito reported the production of small quantities of D(−)-lactic from glucose solution by *R. chinensis* in 1911.

According to Ehrlich (1919), small quantities of L(+)-lactic D(−)-malic acid, and succinic acid, but principally fumaric acid produced by some species of *Rhizopus.*

Takahashi and his associates (1925) demonstrated that as muc 38 per cent of the glucose fermented was converted to D(−)-lactic by certain species of *Rhizopus* in some of their experiments. Vari quantities of acetic, formic, fumaric, D(−)-malic, and succinic acids ethyl alcohol were produced at the same time. In later work, Takah and Asai (1933) showed that four species of *Mucor* produced trace lactic acid from glucose media. Traces of pyruvic and succinic acids likewise found, but ethyl alcohol was the principal product formed.

Miyaji (1930) reported the production of L(+)-lactic and succ acids from glucose media by a new species of *Monilia,* isolated fro commercial culture of soy sauce.

In 1934, results of work carried out by Kanel, in which he use species of *Rhizopus* similar to *R. japonicus,* were published. Yields o much as 38 to 40 per cent of lactic acid, on the basis of the carbohyd consumed, were obtained from invert sugar and from starch. The nutr medium contained 10 per cent sugar and 4 per cent calcium carbon Fumaric acid was occasionally recovered from the older cultures, quantity formed being a function of the conditions of culture and ot factors.

Ward and his associates (1936), in studying the biochemical activi of fungi of the genus *Rhizopus,* found several species that converted g cose to L(+)-lactic acid in the presence of calcium carbonate. T strains, *R. oryzae* 394 and *R. oryzae* Went and Geerligs 395, demonstra particularly good results, yields up to 62 per cent, or greater, be obtained by a surface-culture method.

**Methods of Production.** In general, L(+)-lactic acid may be p duced by selected strains of molds by a surface-culture method or by y-fermentor method. The research of Ward, Lockwood, Tabenkin, Vells indicates the superiority of the rotary-fermentor process over urface-culture process.

e following descriptions are based on the research carried out by ists of the Industrial Farm Products Research Division, Bureau of istry and Soils, U.S. Department of Agriculture.

**face-culture Method.** In this method, the molds are grown in Erlenmeyer flasks, each of which contains 75 ml. of nutrient glucose im. Sterilized calcium carbonate is added to the flasks at the rate or 5 g. per flask at the time of inoculation, for in the absence of m carbonate only a small amount of acid is formed.

*Mold.* Lactic acid has been produced from the following molds: *hizus*, *R. chinensis*, *R. pseudochinensis*, *R. elegans*, *R. oryzae*, *R. osus*, *R. shanghaiensis*, *R. stolonifer*, *R. tritici*, certain species of and at least one species of *Monolia*. Of the foregoing molds, *yzae* Went and Geerligs 395 and *R. oryzae* 394 are outstanding in ability to produce L(+)-lactic acid. The authors have found no s indicating the production of lactic acid by aspergilli, penicillia, aria.

*paration of the Mold Suspenson.* Lockwood, Ward, and May prepared their mold suspension in the following manner: The mold own on sterile, moist bread for at least 4 days at 30°C. in order to e an abundance of spores. Stolons and sporangiospores were placed ube or bottle containing sterile water and shaken vigorously to e the spores. Using aseptic precautions, the stolon mass was then d. An estimate of the spore content of the suspension was made d the size of the inoculum regulated to provide 12.5 to 100 million per 200 ml. Pyrex Erlenmeyer flask containing 75 ml. of nutrient medium.

*Medium.* The composition of the medium used for the production c acid was as shown below.

| | |
|---|---|
| Glucose (commercial grade) | 15 per cent |
| $KH_2PO_4$ | 0.3 g./liter |
| $NH_4NO_3$, $(NH_4)_2SO_4$, or urea, to yield | 0.5 g. nitrogen/liter |
| $MgSO_4 \cdot 7H_2O$ | 0.25 g./liter |

*t of Variations in the Constituents of the Medium.* Lockwood, and May studied the physiology of *R. oryzae* Went and Geerligs ought out many important facts. The optimum concentration of was 15 per cent, determined on the basis of lactic acid formed, cose consumed, and the weight of the mycelia.

best yields of lactic acid were produced when the concentration $PO_4$ was 0.6 g. per liter. Doubling this amount of the salt did ease the yield of lactic acid.

onium chloride, ammonium nitrate, ammonium sulfate, D,L-alanine, nic acid, glycine, peptone, and urea served as satisfactory sources gen. Sodium nitrate failed to produce growth, while sodium nitrite d only a slight amount of growth. Table 172 shows the effect of sources of nitrogen on the metabolism of *R. oryzae*.

TABLE 172. THE EFFECT OF DIFFERENT SOURCES OF NITROGEN * ON THE METABOLISM OF *Rhizopus oryzae* †

| Nitrogen source | Quantity per liter, g. | Weight of mycelium, g. | Glucose consumed, g. | Calcium dissolved |  |  | L(+)-Lactic acid |  | Fumaric acid |  |
|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  | Total, g. | Owing to L(+)-lactic acid, per cent | Owing to fumaric acid, per cent | Weight, g. | Weight yield, ‡ per cent | Weight, g. | Weight yield, ‡ per cent |
| $NaNO_3$ | 3.00 | 0.000 | 0.0 | 0.00 | 0.0 | 0.0 | 0.00 | 0.0 | 0.000 | 0.0 |
| $NaNO_2$ | 2.56 | 0.026 |  |  |  |  |  |  |  |  |
| $NH_4NO_3$ | 2.88 | 0.596 | 8.1 | 1.05 | 86.0 | 3.2 | 4.03 | 49.8 | 0.099 | 1.2 |
| $(NH_4)_2SO_4$ | 2.36 | 0.301 | 6.2 | 0.79 | 95.0 | 3.5 | 3.36 | 54.2 | 0.082 | 1.3 |
| $NH_4Cl$ | 1.98 | 0.370 | 7.2 | 1.05 | 87.5 | ... | 4.13 | 57.4 |  |  |
| Urea | 2.14 | 0.613 | 8.6 | 1.24 | 87.5 | 6.0 | 5.44 | 63.3 | 0.217 | 2.5 |
| D,L-Alanine | 3.30 | 0.350 | 8.3 | 1.26 | 86.0 | 8.6 | 4.87 | 58.7 | 0.312 | 3.8 |
| L-Glutamic acid | 5.46 | 0.356 | 7.1 | 1.08 | 83.0 | 6.6 | 4.05 | 57.0 | 0.206 | 2.9 |
| Glycine | 2.67 | 0.257 | 5.9 | 0.87 | 86.0 | 7.2 | 3.37 | 57.1 | 0.183 | 3.1 |
| Peptone | 3.24 | 0.219 | 8.3 | 1.29 | 85.0 | 5.0 | 4.95 | 59.6 | 0.189 | 2.3 |

* 75 ml. of 15.1 per cent glucose nutrient solution contained 0.25 g. $MgSO_4 \cdot 7H_2O$ and 0.3 g. $KH_2PO_4$ per liter; 5 g. $CaCO_3$ per flask. Duration, 14 days. Temperature, 30°C.

† L. B. Lockwood, G. E. Ward, and O. E. May, *Jour. Agr. Research,* **53**: 849 (1936).

‡ Grams of acid produced divided by grams of glucose consumed.

*ffect of Zinc Sulfate.* When calcium carbonate was a constituent he culture medium, the addition of 10 mg. of zinc, in the form of zinc ate, caused an increase in the weight of mycelium, in the glucose umption, and in the absolute quantity of lactic acid formed by *R. ae* Went and Geerligs 395. The yield of L(+)-lactic acid, calculated rms of grams of acid formed divided by grams of glucose utilized, me progressively smaller as the concentration of zinc was increased, ever. In the absence of calcium carbonate, there was no increase in glucose consumption.

*fect of Other Salts.* Ferric and chromium ions, in both the presence the absence of calcium carbonate, produced no apparent effect on se consumption or lactic acid production.

*mperature.* A temperature of 30°C. is favorable for the production ctic acid. At 40°C., the growth of the mold is more rapid but the of acid is less than at 30°C.

*ubation Period.* Cultures are usually incubated for 16 to 21 days. ffect of the incubation period on results obtained with *R. oryzae* is n in Table 173.

173. THE EFFECT OF THE LENGTH OF THE INCUBATION PERIOD ON THE METABOLISM OF *Rhizopus oryzae* *, ‡

| | | Calcium dissolved | | | L(+)-Lactic acid | | Fumaric acid | |
|---|---|---|---|---|---|---|---|---|
| Weight of mycelium, g. | Glucose consumed, g. | Total, g. | Owing to L(+)-lactic acid, per cent | Owing to fumaric acid, per cent | Weight, g. | Weight yield,† per cent | Weight, g. | Weight yield,† per cent |
| 0.040 | 1.3 | 0.067 | .. | ... | 0.060 | | | |
| 0.039 | 2.0 | 0.24 | 87 | 0 | 0.93 | 46.5 | 0 | 0 |
| 0.220 | 6.3 | 0.85 | 88 | 0 | 3.36 | 53.3 | 0 | 0 |
| 0.889 | 10.5 | 1.28 | 78 | 8.7 | 4.50 | 42.9 | 0.314 | 3.0 |
| 1.032 | 11.2 | 1.44 | 72 | 13.9 | 4.66 | 41.6 | 0.564 | 5.0 |

al. of 15 per cent glucose; 0.25 g. $MgSO_4 \cdot 7H_2O$, 0.3 g. $KH_2PO_4$, and 2.88 g. $_3$ per liter; 5 g. $CaCO_3$ per flask. Temperature, 30°C.

ms of acid produced divided by grams of glucose consumed.

. Lockwood, G. E. Ward, and O. E. May, *Jour. Agr. Research*, **53**: 849 (1936).

*aric Acid.* Production of fumaric acid by *R. oryzae* Went and 395 is a variable factor. For example, Lockwood, Ward, and und that a temperature of 40°C. is more favorable for its producan 30°C., that ammonium nitrate ($NH_4NO_3$) is a concentration than 6 g. per liter and high concentrations of glucose inhibit its on, and that it is not produced during the early part of the fermenut only after several days.

*Other End Products.* Acetic, D(−)-malic, and succinic acids an alcohol have been detected in traces, or in small quantities, in som cose media fermented by *R. oryzae.*

*Yields.* Yields of as high as 62 to 67 per cent lactic acid hav obtained by the surface-culture method on the basis of the consumed.

The largest quantity of L(+)-lactic acid is produced just before lation of the mold.

**The Rotary-fermentor Process.** Lactic acid may be produced in 35 hr. (Ward et al., 1938) in rotating aluminum drums of the described in the chapter on The Gluconic Acid Fermentation. This p is superior to the surface-culture process from the standpoint both shorter fermentation period and of the quantity of acid produced the sugar consumed, yields of 70 to 75 per cent of acid being commo

*Procedure.* Spores are produced for inoculation purposes by gro the mold on sterile, moist bread. A special germination medium is i lated to contain 420 million spores per 1.5 liters of the medium. germination medium containing the spores is shaken for 24 hr. at 3 in a 4-liter glass bottle equipped with an outlet tube. By using medium, the composition of which is shown in Table 174, a savin fermentation time is effected.

TABLE 174. SOME MEDIA USED IN LACTIC ACID PRODUCTION *

| Substance | Germination medium, g. | Fermentation medium, g. |
|---|---|---|
| Glucose (91.5 per cent, commercial)..... | 110 | 150 |
| Urea .................................. | 2.0 | 2.0 |
| $KH_2PO_4$ ............................ | 0.60 | 0.60 |
| $MgSO_4 \cdot 7H_2O$ .................. | 0.25 | 0.25 |
| $ZnSO_4 \cdot 7H_2O$ .................. | 0.088 | 0.044 |
| $CaCO_3$ .............................. | 10.0 | † |
| Octadecyl alcohol ..................... | ........ | 0.03 ‡ |
| Distilled water ....................... | to 1 liter | to 1 liter |

* G. E. Ward, L. B. Lockwood, B. Tabenkin, and P. A. Wells, *Ind. Eng. Chem.,* 1233 (1938). Copyright 1938 by the American Chemical Society and reprinted permission of the copyright owner.

† 200 g. to each 3-liter portion—sterilized separately.

‡ Dissolved in 1.7 ml. ethanol (added to prevent excessive foaming of the mentation medium during the rotation of the drum).

A 3-liter portion of the fermentation medium is inoculated with 250 of the germinated spore culture and placed in the aluminum drum. drum is operated at a gauge pressure of 5 lb. per sq. in. and a rotati speed of 13 r.p.m. Air, measured at the exit, flows through at the rate 150 ml. per min., while the temperature is maintained at 35°C.

On account of the limited solubility of calcium lactate and in view the higher yields obtained by this method, it is necessary to use a som ıt lower concentration of glucose than is the case in the surface-culture hod. When a precipitate of calcium lactate settles out, forming a te mass, fermentation is much inhibited. If the settling occurs before he sugar is utilized, it is particularly objectionable. By using 13.per , or less, glucose, satisfactory results are usually obtained.

*esults of a Typical Experiment.* The following data are quoted to trate the result of a typical fermentation carried out by the rotating n method, using *R. oryzae* Went and Geerligs:

| | G./100 Ml. * |
|---|---|
| Original glucose concentration | 13.3 |
| Glucose consumed | 12.8 |
| Ethyl alcohol produced | 0.62 |
| Calcium in solution | 2.245 |
| Lactic acid equivalent to dissolved calcium | 10.12 |
| Lactic acid found by analysis | 9.66 |
| Acidity caused by lactic acid, per cent | 95.4 |
| Yield of L(+)-lactic acid, based on glucose consumed, per cent | 75.5 |

. E. Ward, L. B. Lockwood, B. Tabenkin, and P. A. Wells, *Ind. Eng. Chem.*, **30**: (1938). Copyright 1938 by the American Chemical Society and reprinted by ssion of the copyright owner.

e rate at which glucose was con- d in this experiment is indicated in 110. It will be observed that there but little consumption of glucose g the first 15 hr., but during the rest e experiment the sugar was con- l at a fairly rapid rate.

**piration and Lactic Acid Produc-** Waksman and Foster (1938) have d out research concerning respira- ınd lactic acid production by a s of the genus *Rhizopus.* They suggested reactions to explain the nism of these important processes.

**antages of Mold Process.** Al- ı L(+)-lactic acid, a physiologi- mportant acid (known as "sarco- acid," also) is not produced ercially by molds at present, this s has, as pointed out by Ward and ociates, several apparent advan- Owing to the fact that urea is s the source of nitrogen, a lactic ee from color and of relatively ırity may be produced. The fer- ion is rapid, and salts of lactic e easily recovered. Furthermore, ocess provides a source of L(+)-

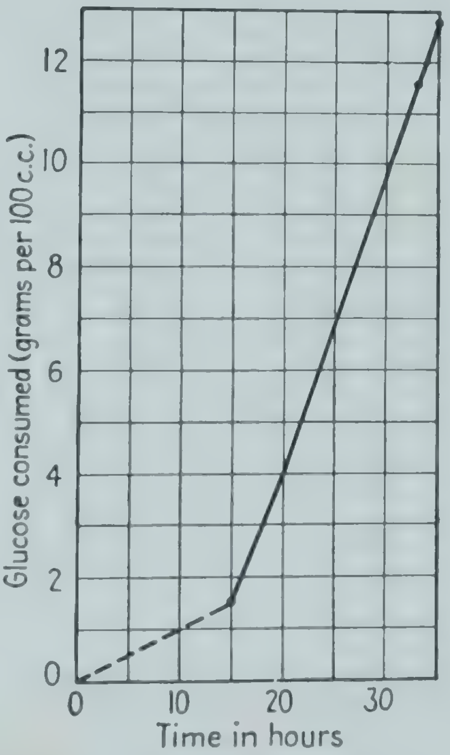


FIG. 110. Course of a typical L(+)-lactic acid submerged fermentation induced by *Rhizopus oryzae.* [*Courtesy of G. E. Ward, L. B. Lockwood, B. Tabenkin, and P. A. Wells, Ind. Eng. Chem.,* **30**: 1233 (1938). *Copyright* 1938 *by the American Chemical Society and reprinted by permission of the copyright owner.*]

lactic acid, which may be used for the preparation of crysta L(+)-lactic acid.

### References on Production of Lactic Acid by Molds

Chrzaszcz, T.: Die "Chinesiche Hefe," *Mucor cambodja,* eine neue technische H nebst einigen Beobachtungen über *Mucor rouxii, Zentr. Bakt. Parasitenk.,* A **7**: 326 (1901).

Ehrlich, F.: Über Fumarsäure—Gärung des Zuckers, *Ber.,* **52**: 63 (1919).

Eijkmann, C.: Mikrobiologisches Untersuchungen über die Arrakfabrikati Batavia, *Zentr. Bakt. Parasitenk.,* Abt. I, **16**: 97 (1894).

Kanel, E.: Lactic Acid Formation in the Culture of a Fungus of the *Rhizopus* Sp *Mikrobiologiia,* **3**: 259 (1934).

———: Comparative Study on the Mechanism of the Respiratory Process o Closely Related Strains of *Rhizopus nigricans* in Connection with the Differ tion of the Thallome and the Changes in External Conditions, *Mikrobiol* **4**: 636 (1935).

Lockwood, L. B., G. E. Ward, and O. E. May: The Physiology of *Rhizopus o Jour. Agr. Research,* **53**: 849 (1936).

——— and A. J. Moyer: The Production of Chemicals by Filamentous Fungi, *B Rev.,* **4**: 140 (1938).

Miyaji, K.: Notiz über *"Monilda tamari"* Kagami Kenkyu Hokoku, *Research Gifu Imp. Coll. Agr.,* **10**: 1–5 (1930).

Saito, E.: Ein Beispiel von Milchsäurebildung durch Schimmelpilze, *Zentr. Parasitenk.,* Abt. II, **29**: 289 (1911).

Takahashi, T., and T. Asai: On the Products of Fermentation by *Mucor* Group The Products of Fermentation in Presence of Ca-Carbonate, *Zentr. Bakt. Pa tenk.,* Abt. II, **89**: 81: 1933.

——— and K. Sakaguchi: Studies on the Acids Formed by *Rhizopus* Species, J *Agr. Chem. Soc. Japan,* **1**: 46 (1925).

———, ———, and T. Asai: Studies on the Acids Formed by *Rhizopus* Species. Formation of Ethyl Alcohol from Tartaric or Fumaric Acid, *Bull. Agr. Ch Soc. Japan,* **2**(5): 61 (1926).

Waksman, S. A., and J. W. Foster: Respiration and lactic acid production by a fu of the genus *Rhizopus, Jour. Agr. Research,* **57**: 873–900 (1938).

——— and I. J. Hutchings: Lactic Acid Production by Species of *Rhizopus,* J *Am. Chem. Soc.,* **59**: 545–547 (1937).

Ward, G. E., L. B. Lockwood, O. E. May, and H. T. Herrick: Studies in the Ge *Rhizopus.* I. The Production of Dextro-lactic Acid, *Jour. Am. Chem. Soc.,* 1286 (1936).

———, ———, B. Tabenkin, and P. A. Wells: Rapid Fermentation Process Dextro-lactic Acid, *Ind. Eng. Chem.,* **30**: 1233 (1938).

### Some Patents

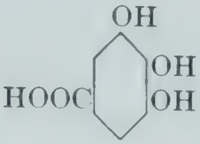
Boullanger, E.: Improvements in the Manufacture of Lactic Acid, British Pat 13,439, 1899; also German Patent 118063, 1901.

Ward, G. E., L. B. Lockwood, and O. E. May: Fermentation Process for the Ma facture of Dextro-lactic Acid, U.S. Patent 2,132,712, Oct. 11, 1938.

## CHAPTER 35

# THE GALLIC ACID FERMENTATION

llic acid (trihydroxybenzoic acid) has the following structural ula:



curs naturally in gall nuts, sumac, walnuts, tea, and other plants. prepared chemically by the hydrolysis of tannin.

ieele discovered gallic acid when he was studying the effect of a on a water infusion of gall nuts (1787, or earlier). Gall nuts are n tannin compounds and are produced principally on species of the ree and the sumac, as the result of insect injuries.

n Tieghem (1867) carried out classical studies in connection with allic acid fermentation. (The interested reader should refer to his publications. To Van Tieghem, credit is due for being the first man ablish the importance of the aspergilli in the biochemical field, his having been done at a time when very little was known of modern ulture methods.) Van Tieghem identified *Aspergillus niger* as the ninating mold in the fermentation. The *Penicillium glaucum* group ds also possessed the ability to ferment tannin to gallic acid. Van m showed that air was essential for mold growth and for the suc- production of gallic acid from moist gall nuts or tannin liquor.

ne of the oldest methods used for the production of gallic acid by tation, the substances containing tannin were piled up in heaps oistened with water. Molds developed throughout the heap, which irred occasionally and maintained at a temperature of approxi- 30°C. After a fermentation period of about a month the gallic as leached from the heap.

ent-day methods make use of clear tannin extracts, which are ed and then inoculated with pure cultures of a species of *Asper-* The solution is agitated by mechanical means, and air is blown 1 it. The temperature of the fermentation is carefully controlled. is of the mash is made occasionally in order that the fermentation stopped promptly after the tannin has been completely utilized. conversion of tannin to gallic acid is actually brought about by of the enzyme "tannase." Fernbach and Pottevin, each working dently, demonstrated that *A. niger* produced tannase in the presence of tannin and nutrient materials. Using the mold-free enz was shown that the fermentation would proceed independently of tl

Knudson discovered that he could cause a progressive increas tannase content of *A. niger* by replacing the sugar in Czapek's with tannic acid. He obtained a maximum production of tanna the 10 per cent of sugar in the solution had been replaced with 2 of tannic acid.

Gallic acid has several important uses in industry. It has be in the manufacture of gallocyanin, a dye; it is used as a basic mat the production of alizarin brown. Inks are made from it. Co tion with sulfuric acid yields hexahydroxyanthraquinone. In co tion, gallic acid finds use as a skin-disease remedy.

### References on the Gallic Acid Fermentation

Calmette, A.: Verfahren zur Umwandlung von Tannin in Gallussäure, Patent 129164, Mar. 1, 1902.

Duclaux, E.: Chimie biologique, in E. Frémy, "Encyclopédie Chimique," t. 9, Paris, 1883.

Fernbach, A.: Sur la tannase, *Compt. rend.*, **131**: 1214–1215 (1900).

Knudson, L.: Tannic Acid Fermentation, *Jour. Biol. Chem.*, **14**: 159–202 (191

May, O. E., and H. T. Herrick: Some Minor Industrial Fermentations, *In Chem.*, **22**: 1172–1176 (1930).

——— and ———: Production of Organic Acids from Carbohydrates by Fer tion, *U.S. Dept. Agr., Circ.* 216, May, 1932.

Porter, J. R.: "Bacterial Chemistry and Physiology," John Wiley & Sons, Inc York, 1946.

Pottevin, H.: La tannase, diastase dedoublant l'acide gallotannique, *Compt.* **131**: 1215–1217 (1900).

Scheele, K.: Über das Wesentliche Galläpfelsalz, *Crell's Chem. Ann.*, **1**: 3–12

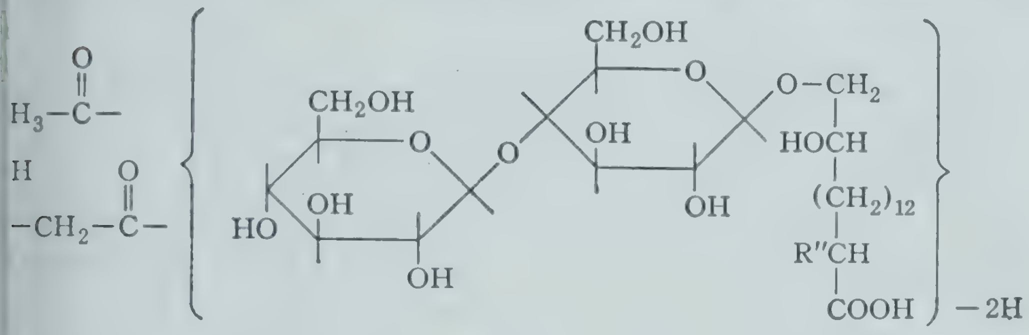
Thom, C., and M. B. Church: "The Aspergilli," The Williams & Wilkins Co Baltimore, 1926.

Van Tieghem, P. E. L.: Fermentation gallique, *Ann. sci. nat. Botan.*, 1867, s. p. 240.

———: Sur la fermentation gallique, *Compt. rend.*, **65**: 1091–1094 (1867).

# USTILAGIC ACID

ilagic acid is a crystalline mixture of monoacidic beta-cellobiolipids ting of about 2 parts of I to 1 part of II (Lemieux, 1951; Lemieux 1951). The components of the mixture have not been separated.



I, $R' = CH_3(CH_2)_2^-$, $R'' = H$

II, $R' = CH_3(CH_2)_4^-$, $R'' = OH$

lagic acid is produced by the corn-smut fungus, *Ustilago zeae* ins, 1950). It is of special interest because it, or some of its derivanay be used as the starting material for the synthesis of rare and ive perfumes (Lemieux, 1953).

**erties.** Ustilagic acid is insoluble in water and precipitates from mentation medium in crystalline form. It is readily soluble in iol, 2,3-butanediol, pyridine, and 1,2-propanediol and sparingly in acetone, butanol, and ethanol (Lemieux et al., 1951).

adation of ustilagic acid by refluxing with methanolic hydrochloric ves rise to substantially quantitative yields of the methyl esters acids: 15D,16-dihydroxyhexadecanoic acid and 2D,15D,16-trihyiexadecanoic acid, which are known as "ustilic acids" and whose l esters are insoluble in petroleum ether; and 3-hydroxy-L-hexanoic nd 3-hydroxy-L-octanoic acid, whose methyl esters are soluble in um ether (Lemieux, 1951; Lemieux and Charanduk, 1951; Lemieux 951).

**uction.** *U. zeae* (PRL-119), known as the "corn-smut fungus," d by Haskins (1950, 1955), Thorn and Haskins (1951), and Rox-Spencer, and Sallans (1954) in their production studies.

fungus may be isolated from smutted corncobs by shaking the ver the top of potato dextrose or other suitable agar exposed in ishes, by incubating the seeded petri dishes for about 1 week at

28 to 30°C., and by selecting pure cultures. The fungus exhibit biotic activity which inhibits the growth of most contaminating mi (Haskins, 1950). The pure cultures may be maintained on shor slants under sterile mineral oil.

A medium containing 0.06 per cent urea, 0.06 per cent yeast e 5 per cent glucose, and 1.5 per cent agar in a mineral-base so (0.1 per cent monobasic potassium phosphate, 0.04 per cent magn sulfate crystals, and 0.003 per cent ferrous sulfate crystals in di water) was found by Haskins to be excellent for the maintenance smut cultures.

Roxburgh, Spencer, and Sallans (1954) discussed factors which enced the production of ustilagic acid by *U. zeae* (PRL-119) in merged-culture fermentation in 5-liter and in 200-gal. fermentors. basal medium used by them contained the following ingredients per

| | G. |
|---|---|
| $KH_2PO_4$ | 1.0 |
| $MgSO_4 \cdot 7H_2O$ | 0.4 |
| $FeSO_4 \cdot 7H_2O$ | 0.03 |
| Urea | 1.2 |
| $CaCO_3$ | 1.7 |
| Molasses, sugar-beet | 0.6 |

Glucose monohydrate was added to the basal medium in concentrat of 50 to 180 g. per liter.

The culture for inoculation purposes was grown for 48 hr. in 500- shake flasks containing 100 ml. of the basal medium and 7.5 per cent glucose monohydrate or in 5-gal. fermentors. The production medi was inoculated with 5 per cent by volume of the seed culture.

The fermentations were carried out at 30°C. with an aeration le equivalent to a sulfite oxidation rate of over 80 mM. of oxygen per li per hour. Maximum efficiency (the ratio of the carbon recovered ustilagic acid to the carbon added as glucose) was found to be about per cent when a 10 per cent glucose monohydrate concentration and aeration rate of 82 mM. of oxygen per liter per hour were used. This w equivalent to the production of 0.23 lb. of ustilagic acid per pound anhydrous glucose. Yields of greater than 22 g. of ustilagic acid per li of medium were obtained in 40 to 50 hr.

Methods of recovering ustilagic acid and useful degradation produ made from it have been described by Roxburgh and associates.

**Synthetic Musks.** Lemieux (1953) discussed the use of ustilagic ac as a starting material for the making of macrocyclic musks valuable the perfume industry.

## Some References concerning the Biochemistry of the Ustinaginales and Ustilagic Acid

### I. Biochemistry of the Ustinaginales

Boothroyd, B., J. A. Thorn, and R. H. Haskins: X. The Biosynthesis of Ustilag Acid, *Can. Jour. Biochem. and Physiol.*, **33**: 289–296 (1955).

———, ———, and ———: XII. Characterization of Extracellular Glycolipids Pr duced by *Ustilago* Sp., *Can. Jour. Biochem. and Physiol.*, **34**: 10–14 (1956).

kins, R. H.: I. Preliminary Cultural Studies of *Ustilago zeae*, *Can. Jour. Research*,
C, **28**: 213–223 (1950).

— and J. A. Thorn: VII. Antibiotic Activity of Ustilagic Acid, *Can. Jour. Botany*,
**29**: 585–592 (1951).

—, J. A. Thorn, and B. Boothroyd: XI. The Metabolic Products of *Ustilago zeae*
in Submerged Culture, *Can. Jour. Microbiol.*, **1**: 749–756 (1955).

ieux, R. U.: III. The Degradation Products and Proof of the Chemical Hetero-
geneity of Ustilagic Acid, *Can. Jour. Chem.*, **29**: 415–425 (1951).

— and R. Charanduk: VI. The Acyl Groups of Ustilagic Acid, *Can. Jour. Chem.*,
**29**: 759–766 (1951).

— and J. Giguere: IV. The Configuration of Some β-Hydroxy-acids and the Bio-
reduction of β-Ketoacids, *Can. Jour. Chem.*, **29**: 678–690 (1951).

—, J. A. Thorn, C. Brice, and R. H. Haskins: II. Isolation and Partial Character-
zation of Ustilagic Acid, *Can. Jour. Chem.*, **29**: 409–414 (1951).

n, J. A., and R. H. Haskins: V. Factors Affecting the Formation of Ustilagic
Acid by *Ustilago zeae*, *Can. Jour. Botany*, **29**: 403–410 (1951).

## II. Other

ins, R. H.: Ustilagic Acid and Method of Preparing the Same, U.S. Patent
,698,843, 1955.

eux, R. U.: Perfumery Synthetic Musks from a Metabolic Product of Corn
mut in Artificial Culture, *Perfumery Essent. Oil Record*, **44**: 136–139 (1953).

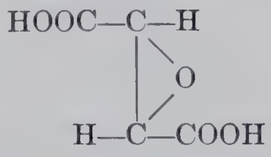
urgh, J. M., J. F. T. Spencer, and H. R. Sallans: Submerged Fermentation.
actors Affecting the Production of Ustilagic Acid by *Ustilago zeae*, *Jour. Agr.
ood Chem.*, **2**(22): 1121 (1954).

## CHAPTER 37

# SOME OTHER ACIDS PRODUCED BY MOLDS

### *TRANS*-L-EPOXYSUCCINIC ACID

*Trans*-L-epoxysuccinic acid, also known as "ethylene oxide-alpha dicarboxylic acid," possesses the following structural formula:

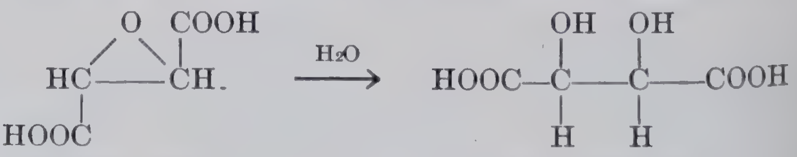


The production of this acid by microbiological means was first rep by Sakaguchi and associates in 1939. They found that *Monilia fo* nov. sp. (stocked as *Paecilomyces varioti* NRRL 1282 by the Nor Utilization Research and Development Division of the U.S. Depart of Agriculture) and *Penicillium viniferum* nov. sp. (stocked as *P. verr* *latum* NRRL 1009) formed this acid. Birkinshaw et al. (1945) desc its production by *Aspergillus fumigatus* NRRL strain 1986.

In 1955, Martin and Foster discussed the production of *trans*-L-ep succinic acid by a number of fungi and its microbiological conversi *meso*-tartaric acid. They used a medium which contained 3 g. KH 6 g. $Na_2HPO_4$, 1 g. $NH_4Cl$, 0.12 g. $MgSO_4\cdot 7H_2O$, 5 mg. $ZnSO_4\cdot 5$ 10 mg. $FeSO_4$, 1 liter distilled water, and 3 per cent glucose or 1.5 per ethanol. Best yields were obtained when 0.5 g. (wet weight) of preg mycelium was used to seed 40 ml. of medium. The cultures were incub on a reciprocating shaker at 30°C. Yields of 21 per cent and 8.7 per of *trans*-L-epoxysuccinic acid were produced in 4 days by *A. fumig* from media containing 3 per cent glucose and 1.5 per cent eth respectively.

Martin and Foster suggested the possibility that malic acid may b immediate precursor of *trans*-L-epoxysuccinic acid.

They demonstrated that several fungi, bacteria, and a yeast, isol from the soil, were able to utilize *trans*-L-epoxysuccinic acid as a source of carbon and that cell-free extracts of bacteria were able to con this acid to *meso*-tartaric acid in accordance with the following reacti



*trans*-L-Epoxysuccinic acid

*meso*-Tartaric acid

he enzyme which catalyzes this reaction was named "*trans*-succinice- de hydrolase."

## THE ITATARTARIC ACID FERMENTATION

1945. Stodola, Friedkin, Moyer, and Coghill, of the Northern onal Research Laboratory, reported on the production of itatartaric by an ultraviolet-induced mutant of *Aspergillus terreus*. The mold grown in a medium that contained 220 g. of glucose, 0.5 g. of $O_4 \cdot 7H_2O$, 0.05 g. of KCl, 0.418 g. of $H_3PO_4$, 0.022 g. of $ZnSO_4 \cdot 7H_2O$, of 0.5 *N* HCl, 8 ml. of 0.5 *N* $HNO_3$, 4 g. of $NH_4NO_3$, 1 g. of corn- liquor, and distilled water to make 1 liter. The medium was dis- d in 750-ml. amounts in 3-liter flasks and inoculated with *A. terreus* L 265.S14. The flasks were incubated at 30°C. for 10 days. From liters of the culture liquor, after the removal of the mycelial mats, btained 134.9 g. of crystalline itaconic acid. A mixture of itatartaric nd its lactone accounted for 5.8 per cent of the total weight of acid nulated during the fermentation. Details of the procedure followed lating and identifying the products may be obtained by reference to iginal report.

### Some References concerning *Trans*-L-epoxysuccinic Acid

haw, J. H., A. Bracken, and H. Raistrick: 73. Metabolic Products of *Asper- lus fumigatus* Fresenius, *Biochem. Jour.*, **39**: 70–72 (1945).

, W. R., and J. W. Foster: Production of Trans-L-epoxysuccinic Acid by Fungi Its Microbiological Conversion to Meso-tartaric Acid, *Jour. Bact.*, **70**: 405– (1955).

hi, K., T. Inoue, and S. Tada: On the Production of An Ethylene-$\alpha$-$\beta$-di- oxylic Acid by Molds, *Zentr. Bakt. Parasitenk.*, Abt. II, **100**: 302–307 (1939).

### Reference concerning Itatartaric Acid

F. H., M. Friedkin, A. J. Moyer, and R. D. Coghill: Itatartaric Acid, a abolic Product of an Ultraviolet-induced Mutant of *Aspergillus terreus*, *Jour. . Chem.*, **161**: 739–742 (1945).

## CHAPTER 38

# MANNITOL PRODUCTION BY MOLDS

Mannitol ($CH_2OH\cdot(CHOH)_4\cdot CH_2OH$), an alcohol, occurs na in manna, an exudate from certain plants, prominent among whic manna ash tree, *Fraxinus ornus.* It is formed as a fermentation from fructose by certain bacteria.

Braconnot (1811) and Vauquelin (1813) reported that mannit found to be a constituent of the tissue of some of the higher fungi. nitol has since been found in the mycelium of some of the lower f well. As such, it is regarded as a reserve food product rather tha product of fermentation.

Several fungi, however, produce mannitol as a fermentation p from nutrient sugar solutions. Mannitol is produced from glucos Czapek-Dox solution by certain white aspergilli (Birkinshaw 1931), by *Aspergillus elegans*, by *A. nidulans*, by *Helminthosp* *geniculatum*, by a species of *Clasterosporium*, by *Byssochlamys fulv* by *Penicillium chrysogenum*. It is produced from sucrose, togethe itaconic acid, by *A. itaconicus*, and from glycerol by molds of the *A.* *cus* group. Ethyl alcohol is formed by two of the foregoing fungi, na *H. geniculatum* and the species of *Clasterosporium*, as one of the products (Birkinshaw and Raistrick, 1931). Other products are f in small quantities by some of these fungi, such as glycerol, acetalde succinic acid, and malic acid.

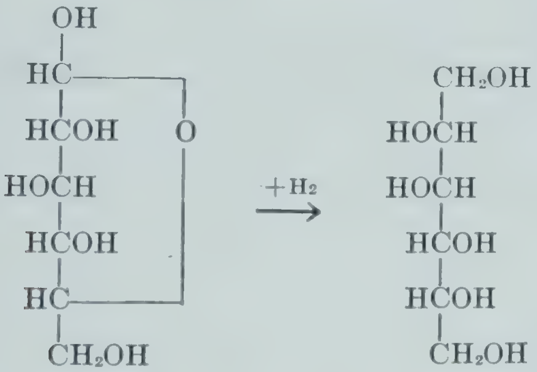
Pruess and his associates (1932) have isolated mannitol from *A. f* and *A. oryzae*.

Yields of as much as 50 per cent mannitol, based on the sugar uti have been produced by a white species of *Aspergillus* (Thom 4640 from the Czapek-Dox solution of glucose (2 g. of $NaNO_3$, 1 g. of KH 0.5 g. of KCl, 0.5 g. of $MgSO_4\cdot 7H_2O$, 0.01 g. $FeSO_4\cdot 7H_2O$, 50 g. of cose, and water to 1,000 ml.).

Two other species of white aspergilli, a strain of *A. elegans* and strains of *A. nidulans*, were investigated by Birkinshaw and his as ates. The metabolic solutions were incubated at 23 or 24°C. usu for periods of 2 to 10 weeks. Control of aeration was found to be im tant. Higher yields were obtained by restricting the air supply. being effected by keeping the flasks closed to all air supply except small amounts of sterilized air that were passed through the flasks du a 30-min. period once a day. Unrestricted aeration resulted in l yields, for the "water-soluble product" formed was used up as the a able glucose disappeared. Some of the highest yields were obtained exhausted metabolism solutions were replaced with fresh nutrient cose solutions. As a result of using the mycelia over again, shorter nentation periods were required.

'he same strain of *Aspergillus* (Coyne and Raistrick, 1931) produced ds of about 35 per cent D-mannitol from glucose, mannose, and galac- on the basis of the sugar utilized. A smaller yield of D-mannitol was ined from xylose, a pentose. The yield from arabinose was poorer that from xylose. No mannitol was produced from fructose, which adily fermented by some bacteria and reduced by chemical means to annitol.

*yssochlamys fulva*, Olliver and Smith (Raistrick and Smith, 1933), scomycete and a cause of spoilage in processed fruits, produces man- from glucose to the extent of about 30 per cent, on the basis of the r consumed, when grown in the Czapek-Dox solution. The inoculated ions were incubated at 24°C. Byssochlamic acid ($C_{18}H_{20}O_6$; m.p. 5°C.) was obtained in yields of about 0.5 per cent along with mannitol. e following equation represents the formation of D-mannitol from cose:



D-Glucose D-Mannitol

mechanism by which D-mannitol is produced from the pentoses is own at present.

## References on Mannitol Production

ıaw, J. H.: Biochemistry of the Lower Fungi, *Biol. Rev.*, **12**: 357 (1937).
J. H. V. Charles, A. C. Hetherington, and H. Raistrick: Studies in the Bio-
mistry of Micro-organisms. IX. On the Production of Mannitol from Glu-
e by Species of *Aspergillus*, *Trans. Roy. Soc. London*, **220B**: 153 (1931).
and H. Raistrick: Studies in the Biochemistry of Micro-organisms. XVII.
Products of Glucose Metabolism Formed by Various Species of Fungi, *Trans.*
*y. Soc. London*, **220B**: 331 (1931).
, A.: "The Chemistry of Micro-organisms," Pitman Publishing Corporation,
York, 1955, pp. 291–293.
ot, H.: Sur la nature des champignons, *Ann. chim.*, **79**: 265 (1811).
Nouvelles recherches analytiques sur les champignons pour servir de suite à
s qui ont été insérées dans les tons, *Ann. chim.*, **87**: 237 (1813).
uck, P. W.: Recent Developments in the Biochemistry of Moulds, *Jour.*
*Chem. Ind.*, **55**: 55T (1936).
F. P., and H. Raistrick: Studies in the Biochemistry of Micro-organisms.
On the Production of Mannitol from Hexoses and Pentoses by a White
es of *Aspergillus*, *Biochem. Jour.*, **25**: 1513 (1931).

Kinoshita, K.: Über die produktion von Itaconsäure und Mannit durc Schimmelpilz, *Aspergillus itaconicus, Acta Phytochim. Japan,* **5**: 27

Pruess, L. M., W. H. Peterson, and E. B. Fred: Isolation and Id Ergosterol and Mannitol from *Aspergillus fischeri, Jour. Biol. C* (1932).

Raistrick, H., and G. Smith: Studies in the Biochemistry of Mi XXXV. The Metabolic Products of *Byssochlamys Fulva* Olliver *Biochem. Jour.,* **27**: 1814 (1933).

——— and W. Young: Studies in the Biochemistry of Micro-organis Estimation of Mannitol in Fermentation Solutions, *Trans. Roy.* **220B**: 173 (1931).

Vauquelin: Expériences sur les champignons, *Ann. chim.,* **85**: 1 (1813).

Yamasaki, I., und M. Shimomura: Über die *d*-Mannitbildung aus Gl Schimmelplize der *Aspergillus-glaucus*-Gruppe, *Biochem. Zeit.,* **291**:

## CHAPTER 39

# PRODUCTION OF MUSHROOM MYCELIUM BY THE SUBMERGED-CULTURE PROCESS

ommercial mushrooms are popular in this country and Europe. They
used in soups and sauces, particularly for flavoring purposes. About
million lb. is produced annually by the conventional method in the
ted States (Humfeld, 1950–1951).

n 1947, Humfeld suggested that the mycelium of mushrooms might
have food value and discovered that certain strains of the mushroom
*ricus campestris* may be grown with agitation and aeration in sub-
ged culture. Details of the method have been described by Humfeld
Sugihara (1948–1954). Another process, together with a procedure
btaining the essence from *A. campestris*, has been patented by Szuecs
0). Block et al. (1953) discussed experiments in which *A. blazei*, a
mushroom in Florida, was grown in submerged culture in orange
, citrus press water, and synthetic media. The product compared
rably with some foods rich in amino acids and vitamins of the B com-
according to the report.

e production of mushroom mycelium by a submerged-culture process
ars to be not too difficult to carry out. However, the problem lies in
ct that the mushroom mycelium so produced is lacking in true mush-
flavor. Should a means be developed for producing mycelium with
h of the true mushroom essence, then the process holds good promise
roducing large amounts of the mycelium in a comparatively short
d of time.

**ngi Studied.** Mushrooms are fungi which are classified as *Basidio-
tes*. Species studied by Sugihara and Humfeld (1954) are listed
ble 175.

ree strains of *A. campestris* were isolated by Humfeld and Sugihara
more than 40 cultures tested. These strains designated as M5, a
variety, M16, a cream variety, and M28, a white variety, were
le of being grown in agitated and aerated media. They produced
led "secondary spores," and their hyphae were much more slender
ongated than those of the other isolates.

**duction.** *Method of Humfeld and Sugihara.* A nitrogen-free basal
n of the composition shown at the top of page 649 was used. Urea
r cent by weight) solution, sterilized by passage through a Seitz
was added at the rate of 15 ml. per liter to the nitrogen-free basal
n after the pH of the latter had been adjusted to 4.5 with sodium

Table 175. Mushroom Species Grown in Submerged Culture on Sy
Medium *

| Species | Culture no. † | Source ‡ | Growth characteristics | ma g. |
|---|---|---|---|---|
| *Agaricus campestris* (white var.) | M5; NRRL 2334 | F | Dispersed, spores present | |
| *A. campestris* (cream var.) | M16; NRRL 2335 | H | Dispersed, spores present | |
| *A. campestris* (white var.) | M28; NRRL 2336 | G | Dispersed, spores present | |
| *A. placomyces* | M86 | A | Dispersed, spores present | |
| *A. rodmanii* | M21 | B | Pellet-like colonies | |
| *Armillaria mellea* | M6 | E | Dispersed, spores present | |
| *Cantharellus cibarius* | M83; NRRL 2370 | A | Pellet-like colonies | |
| *Collybia umbulata* | M82 | A | Pellet-like colonies | |
| *C. velutipes* | M70; NRRL 2367 | D | Pellet-like colonies | |
| *Coprinus comatus* | M46 | B | Dispersed, spores present | |
| *C. comatus* | M67 | D | Dispersed, spores present | |
| *Hebeloma sinapizans* | M84 | A | Pellet-like colonies | |
| *Lepiota naucina* | M73; NRRL 2368 | C | Pellet-like colonies | |
| *L. procera* | M44 | B | Pellet-like colonies | |
| *L. rachodes* | M76 | A | Dispersed, spores present | |
| *Lycoperdon umbrinum* | M85; NRRL 2372 | A | Pellet-like colonies | |
| *Morchella* sp. | M34 | C | Pellet-like colonies | |
| *M. crassipes* | M37; NRRL 2369 | C | Pellet-like colonies | |
| *Pleutotus ostreatus* | M69; NRRL 2366 | B | Pellet-like colonies | |
| *Polyporus sulphureus* | M72 | D | Pellet-like colonies | |
| *Psilocybe* sp. | M77 | A | Pellet-like colonies | |
| *Schizophyllum commune* | M71 | D | Pellet-like colonies | |
| *Tricholoma nudum* | M81; NRRL 2371 | A | Dispersed, spores present | |

---

* T. F. Sugihara and H. Humfeld, *Appl. Microbiol.*, 2(3): 170 (1954). Medium position Humfeld and Sugihara (1949).

† Stocks designated by NRRL are in the Culture Collection of the U.S. Nort Utilization Research and Development Division, U.S. Department of Agricul Peoria, Ill.

‡ Source

| | |
|---|---|
| A | Collected by authors. |
| B | S. Bellici, San Francisco, Calif. |
| C | J. W. Sinden, Pennsylvania State College, State College, Pa. |
| D | R. Davidson, Bureau of Plant Industry, U.S. Department of Agr ture, Beltsville, Md. |
| E | P. A. Ark, Department of Plant Pathology, University of Califo Berkeley, Calif. |
| F | Cave Mushroom Co., Santa Cruz, Calif. |
| G | G. Kennedy, Redwood City, Calif. |
| H | I. J. Hutchings, H. J. Heinz Co., Pittsburgh, Pa. |

| Component | Compound | Elements, mg./liter |
|---|---|---|
| ose .................. | 50 g./liter | |
| PO$_4$ .................. | 0.87 g./liter | K, 250; P, 150 |
| IPO$_4$ .................. | 0.40 g./liter | Mg, 40; P, 100 |
| l$_2$·2H$_2$O .............. | 0.37 g./liter | Ca, 100 |
| )$_4$(2 *N*) .............. | 5.1 ml. | S, 200 |
| e element solution *.... | 20 ml. | Fe, 4; Mn, 4; Zn, 2; Cu, 0.5 |

'he trace element solution contained 0.5 g. $FeCl_3 \cdot 6H_2O$, 0.36 g. $MnCl_2 \cdot 4H_2O$, g. $ZnCl_2$, 0.05 g. $CuSO_4$, and water to make 1 liter.

oxide and it had been sterilized for 20 min. at a steam pressure of ). per sq. in.

ırious nitrogen and carbon sources were found to be satisfactory for lium production. Favorable sources of nitrogen, in addition to urea, ded peptone, amino acids, ammonium hydroxide, monosodium glute, and alfalfa press juice. Good yields were obtained from D-glucose, actose, D-mannose, D-fructose, maltose, D-xylose, L-arabinose, dexmannitol, sucrose, and soluble starch when urea was used as the e of nitrogen. The mushroom was unable to utilize lactose, L-rhamand carboxymethyl cellulose as carbon sources.

search was carried out to determine the effect of the concentration of us elements on the maximum yield and flavor. Results are shown ble 176.

176. CONCENTRATIONS OF ELEMENTS REQUIRED FOR MAXIMUM YIELD AND FOR GOOD FLAVOR *

| Element | Yield, mg./liter | Flavor, mg./liter | Inhibition of growth, mg./liter |
|---|---|---|---|
| n .................. | 1,000.0 | 1,500–2,000 | 3,500 |
| rus ................ | 50.0 | 300–400 | 400 |
| ım ................. | 100.0 | 200–300 | 400 |
| .................... | 50.0 | 200 | 400 |
| ium ................ | 20.0 | 20 | 50 |
| .................... | 0.1 | 2 | 80 |
| .................... | 0.4 | 2 | 20 |

Humfeld and T. F. Sugihara, *Mycologia*, **44**(5): 605 (1952).

found that phosphorus was taken up from the medium in an in excess of that required for maximum growth and was not libagain. Potassium, sulfur, and magnesium were also taken up in ies greater than those required for growth but reappeared later in lium.

nitrogen content of the medium produced a marked effect on the ı content of the dried mycelium. At the end of 5 days, the nitrogen

content of the mycelium varied from 3.2 per cent when the initial r
content of the medium was 500 mg. per liter to 6.9 per cent w
initial nitrogen content of the medium was 3,500 mg. per liter.

The effect of the nitrogen content of the medium on the y
mycelium, pH, refractive index, and invert sugar content is sh
Fig. 111.

*A. campestris* was cultivated at a temperature of 25°C. Air w
plied at the rate of 1 volume per volume of medium per minute w
propagation was carried out in a 20-liter mash. Agitation wa
vided also.

The mycelium may be harvested after 4 to 5 days, but a stronge
of mushroom was obtained when the propagation was continued f
2 days after the sugar in the medium had been consumed.

Yields from *A. campestris* and other mushrooms are shown in Tab

*Method of Block and Associates.* *A. blazei*, an edible wild mus
was grown on a medium of the following composition: 5 per cent g
0.2 per cent potassium nitrate, 0.1 per cent potassium dihydrogen
phate, 0.05 per cent magnesium sulfate heptahydrate, 0.05 per ce
cium nitrate, 0.01 per cent sodium chloride, and a trace of ferrous s

The mushroom was able to utilize organic and inorganic sour
nitrogen, including ammonium salts, nitrates, and urea.

Information on the effect of initial pH on sugar utilized and on
is given in Table 177. It will be observed that pH exerted very
effect on the yield over the range studied.

TABLE 177. EFFECT OF INITIAL pH ON YIELD *

| pH | | Synthetic medium | | Efficiency, % | |
|---|---|---|---|---|---|
| | | Sugar utilized, % (initial sugar 5%) | Mycelium produced, g./100 ml. dry weight | | |
| Initial | Final | | | Fermentation † | P |
| 3.5 | 5.5 | 4.0 | 1.65 | 41 | |
| 4.8 | 5.5 | 4.5 | 1.86 | 41 | |
| 5.5 | 5.4 | 3.5 | 1.52 | 42 | |
| 6.5 | 5.9 | 4.5 | 1.78 | 40 | |
| 7.5 | 5.6 | 4.5 | 1.74 | 39 | |

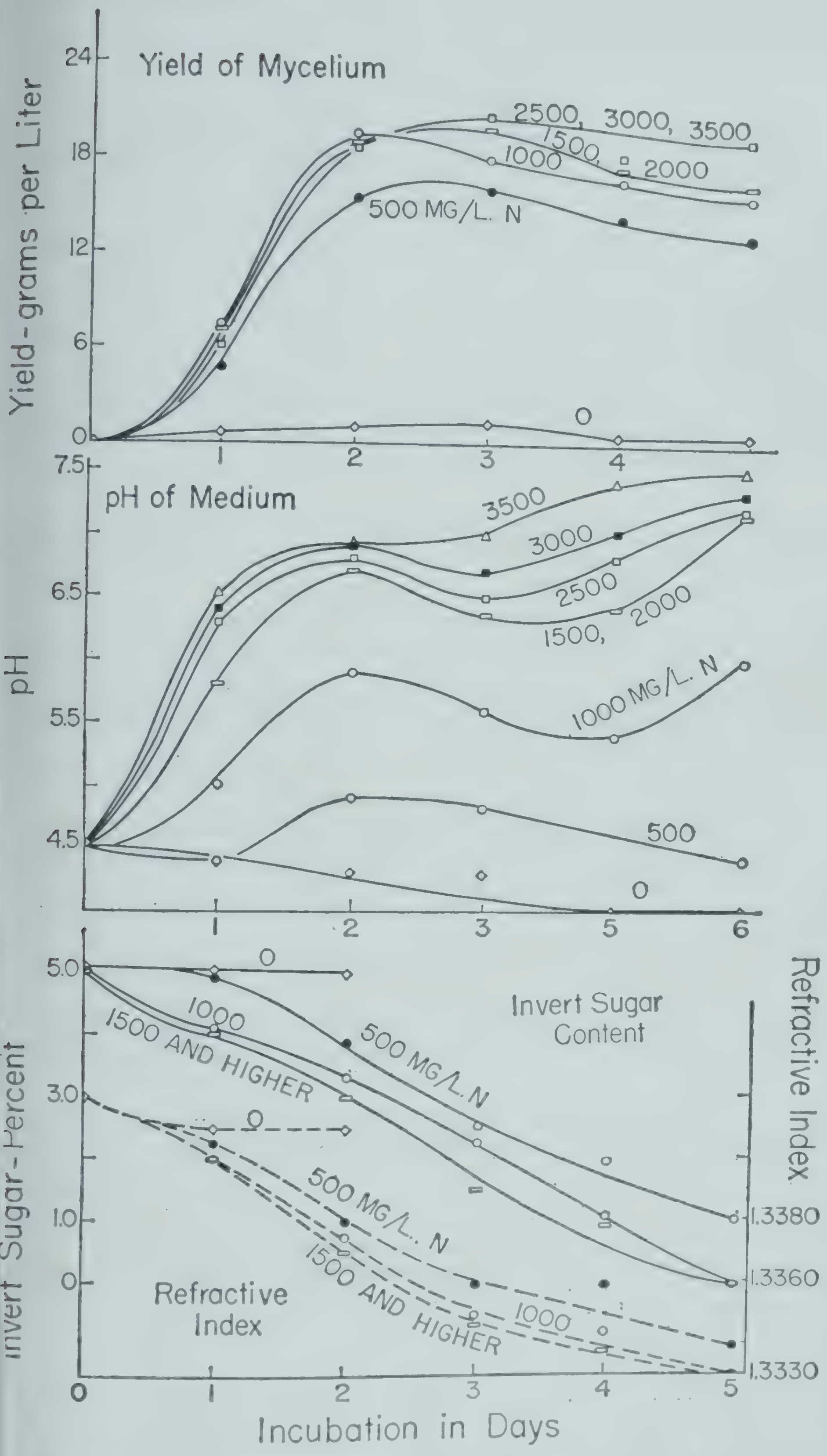
* S. S. Block, T. W. Stearns, R. L. Stephens, and R. F. J. McCandless, *Jour*
*Food Chem.*, 1(14): 890 (1953). Copyright 1953 by the American Chemical S
and reprinted by permission of the copyright owner.

† Based on sugar fermented.

‡ Based on initial sugar.

Data on mycelium production in different media are shown in T
178. Whereas fresh citrus press water was satisfactory for use, that st
in a refrigerator for several weeks was unsatisfactory.

**Flavor.** The greatest single problem, or obstacle, standing in the
of the commercial production of mushroom mycelium by the subme



. Effect of nitrogen concentration on yield of mycelium, pH, refractive index,
ert sugar content of medium. [*Courtesy of H. Humfeld and T. F. Sugihara,*
*ia,* **44**: 605 (1952).]

TABLE 178. MYCELIUM PRODUCTION ON DIFFERENT MEDIA *

| Medium | Sugar utilized, % (initial sugar 6%) | Mycelium produced, g./100 ml. dry weight | Efficiency |
|---|---|---|---|
| | | | Fermentation |
| Citrus press water | 4.3 | 1.88 | 46 |
| Orange juice | 5.0 | 2.06 | 41 |
| Malt extract | 3.9 | 1.98 | 51 |
| Nutrient broth | 5.3 | 2.28 | 43 |
| Corn-steep liquor | 5.2 | 2.66 | 51 |

* S. S. Block, T. W. Stearns, R. L. Stephens, and R. F. J. McCandless, *Jo* *Food Chem.*, 1(14): 890 (1953). Coypright 1953 by the American Chemical and reprinted by permission of the copyright owner.

culture method is the lack of the true mushroom flavor. Sugiha Humfeld (1954) reported that the flavor of *A. campestris* and *rachodes* was pleasant. Block and associates found that gradually ing the mycelium (produced on the synthetic medium) to 75°C. aut the cells and brought out a flavor of the mushroom, which, howev not resemble that of fresh-cooked mushroom. Orange juice impar the mycelium a bitter flavor, which could be removed by washing.

Intensive research on this phase of mushroom mycelium product indicated. It is not known whether the true flavor of the mushro produced by specialized cells of the fruiting body of the commercial uct which are not produced by the submerged mycelium or whether is some essential component, or components, not supplied in the thus far used.

**Nutritive Value.** Study of the accompanying Tables 179 and 180

TABLE 179. COMPARISON OF THE COMPOSITION OF THE MYCELIUM GROWN BY MERGED PROPAGATION AND THAT OF COMMERCIALLY GROWN MUSHROOMS (*A* *campestris*) *

(Calculated to a moisture-free basis)

| | Commercial mushrooms, % † | Mushroom, white variety, % | Mushro brow variety |
|---|---|---|---|
| Protein (N × 6.25) | 37.52 | 35.5 | 45.3 |
| Fat (ether extract) | 1.81 | 3.3 | |
| Nitrogen-free extract (carbohydrate and starches) | 38.19 | 48.8 | |
| Fiber | 10.38 | 6.92 | |
| Ash | 12.00 | 4.59 | 5.2 |
| Ca | 0.023 | 0.12 | 0.1 |
| P | 1.42 | 1.28 | 1.2 |
| Fe | 0.019 | Trace | Tr |

* H. Humfeld and T. F. Sugihara, *Food Technol.*, 3(11): 355 (1949).
† Anderson and Fellers.

.E 180. B VITAMINS OF MUSHROOM MYCELIUM, COMMERCIAL MUSHROOMS, YEAST, AND WHEAT *

| | Mg./100 g. dry weight | | | | | | |
|---|---|---|---|---|---|---|---|
| | Mushroom mycelium | | | Commercial mushroom (*A. campestris*) | Torula yeast | Brewers' yeast | Wheat |
| | *Agaricus blazei* | *A. campestris* | | | | | |
| Vitamin | (*M*) | White | Brown | | | | |
| nin ........ | 0.2 | 0.9 | 0.9 | 1.2 | 0.7– 4.2 | 10.0 | 0.58 |
| lavin ...... | 3.4 | 4.7 | 9.0 | 5.2 | 2.4– 4.7 | 5.8 | 0.16 |
| ı .......... | 14.6 | 19.0 | 29.0 | 58.0 | 37.0–69.0 | 39.0 | 4.8 |
| thenic acid | 6.9 | ... | ... | 23.0 | 10.0–18.0 | | |

S. Block, T. W. Stearns, R. L. Stephens, and R. F. J. McCandless, *Jour. Agr. Chem.*, 1(14): 890 (1953). Copyright 1953 by the American Chemical Society printed by permission of the copyright owner.

that mushroom mycelium is a good source of B vitamins and acids.

## MOREL MUSHROOMS

rel mushrooms are highly prized edible fungi which grow naturally ests. They include such species as *Morchella esculenta, M. conica, nitra esculenta*, and *G. gigas*.

iethod has been developed by the Battelle Memorial Institute (based arch by Yorktown Products) for producing morel mushrooms on a rcial scale.[1] The mushrooms are grown in an aerated, sterilized it medium contained in vats holding 25,000 gal. The mushrooms ed in 72 hr. are full-bodied, stemless, and reputedly of good flavor.

### References on the Submerged Production of Mushroom Mycelium

. S., T. W. Stearns, R. L. Stephens, and R. F. J. McCandless: Mushroom elium Experiments with Submerged Culture, *Jour. Agr. Food Chem.*, **1**(14): 893 (1953).

l. H.: The Production of Mushroom Mycelium (*Agaricus campestris*) in nerged Culture, *Science*, **107**(2780): 373 (1948).

The Production of Mushroom Mycelium, "Yearbook of Agriculture," 1950– pp. 242–245.

nd T. F. Sugihara: Mushroom Mycelium Production by Submerged Propan, *Food Technol.*, **3**(11): 355 (1949).

nd ———: The Nutrient Requirements of *Agaricus campestris* Grown in nerged Culture, *Mycologia*, **44**(5): 605–620 (1952).

3. B.: Principles and Practice of Mushroom Culture, *Econ. Botany*, **8**(1): (1954).

T. F., and H. Humfeld: Submerged Culture of the Mycelium of Various es of Mushroom, *Appl. Microbiol.*, **2**(3): 170–172 (1954).

: Essence of Mushroom and Its Preparation, U.S. Patent 2,505,811, May 2,

atted Mushrooms, *Chem. Week*, **72**(13): 50.

*Eng. News*, May 19, 1958, p. 38.

## CHAPTER 40

# THE PRODUCTION OF FAT BY MOLDS

Browne (1906), Sullivan (1913), Belin (1926), Terroine and (1927), Pearson and Raper (1927), Barber (1929), Rockw O'Flaherty (1931), Pontillon (1933), and others contributed to knowledge of fat formation by molds.

Hesse (1949) reviewed the subject, placing special emphasis contributions made by German scientists.

## THE NATURE OF THE FATS PRODUCED BY MOLDS

**Lipids of Penicillium javanicum van Beijma.** The mold wa vated on a 20 per cent nutrient glucose solution at 30°C., and th mycelium was extracted with a redistilled petroleum ether, yielding cent of oil. Physical and chemical characteristics of the oil are sh accompanying Table 181.

Table 181. Physical and Chemical Characteristics of Oil from *Pe javanicum* *

| | |
|---|---|
| Solidification point, °C. | 6–7 |
| Melting point, °C. | about 15 |
| Specific gravity (25°/25°) | 0.9145 |
| Refractive index (25°) | 1.4680 |
| Acid value | 10.6 |
| Saponification value | 191 |
| Iodine value (Hanus) | 84.0 |
| Reichert-Meissl value | 0.3 |
| Acetyl value | 10.7 |
| Unsaponifiable matter, per cent | 2.00 |
| Saturated acids (corrected), per cent | 30.8 |
| Unsaturated acids (corrected), per cent | 60.8 |
| Melting points of mixed saturated acids, °C. | 52.5 |
| Mean molecular weight of saturated acids | 272 |

* G. E. Ward and G. S. Jamieson, *Jour. Am. Chem. Soc.*, **56**: 973 (1934). Co 1934 by the American Chemical Society and reprinted by permission of the co owner.

Analyses of the unsaturated and saturated fractions are sho Tables 182 and 183.

**Lipids of Aspergillus sydowi.** The alcohol-ether extract of the mycelium of *A. sydowi* contained oleic, linoleic, palmitic, steari *N*-tetracosanic acids; glycerol; and sterols as shown in Table 184.

TABLE 182. ANALYSIS OF UNSATURATED FRACTION *

| | In unsaturated fraction, per cent | In oil | |
|---|---|---|---|
| | | Acid, per cent | Glyceride, per cent |
| acid | 52.1 | 31.7 | 33.2 |
| eic acid | 47.9 | 29.1 | 30.5 |
| al | 100.0 | 60.8 | 63.7 |

. E. Ward and G. S. Jamieson, *Jour. Am. Chem. Soc.*, **56**: 973 (1934).

TABLE 183. ANALYSIS OF THE SATURATED FRACTION

| | In saturated fraction, per cent | In oil | |
|---|---|---|---|
| | | Acid, per cent | Glyceride, per cent |
| ic acid | 69.5 | 21.4 | 22.4 |
| acid | 28.0 | 8.6 | 9.0 |
| osanic acid | 2.5 | 0.8 | 0.8 |
| l | 100.0 | 30.8 | 32.2 |

TABLE 184. COMPOSITION OF THE SIMPLE LIPIDS OF *Aspergillus sydowi* *

| | |
|---|---|
| Fatty acids | 80.8 † |
| Volatile acids (calculated as butyric) | 0.46 |
| Saturated acids | 22.6 |
| Palmitic | 8.8 |
| Stearic | 11.0 |
| *N*-Tetracosanic | 0.9 |
| Unsaturated acids | 52.9 |
| Oleic | 29.6 |
| Linoleic | 16.3 |
| Higher acids | 1.7 |
| Unsaponifiable | 8.18 |
| Total sterols ‡ | 5.36 |
| Glycerol | 4.2 |

I. Strong and W. H. Peterson, *Jour. Am. Chem. Soc.*, **56**: 952 (1934). Copy-
34 by the American Chemical Society and reprinted by permission of the
t owners.
es indicate percentage of the original lipids.
l on the colorimetric sterol determination.

s of **Penicillium aurantio-brunneum.** The mold was grown on
e-inorganic salt solution in the manner described by Peterson and
iates. The mycelial mats (Peterson et al., 1933) were steamed to
the mold, dried at 65°C., ground finely, and extracted with a
ure of alcohol and ether. The dried mycelium yielded 11.6 per
e lipids.

Table 185 shows the approximate composition of the si which were mainly the glycerides of palmitic, stearic, oleic, acids, as determined by Kroeker and associates (1935).

**Fatty Acids of A. nidulans and P. lilacinum.** Table 1 data relating to the fatty acids found in two fat-rich molds. *A. nidulans* possessed a relatively high content of stearic acid

TABLE 185. APPROXIMATE COMPOSITION OF THE SIMPLE LIPIDS

| | Per Cent |
|---|---|
| Total fatty acids | 85.4 |
| Oleic * | 40.2 |
| Linoleic * | 31.2 |
| Palmitic † | 8.6 |
| Stearic † | 5.3 |
| Unsaponifiable | 4.5 |
| Ergosterol | 1.9 |
| Glycerol | 3.1 |

* Calculated from the weight and iodine number of the unsaturated acic

† Calculated from the weight and neutral equivalent of the crude satu

TABLE 186. THE COMPONENT FATTY ACIDS OF THE FATS OF *Aspergillus n*
*Penicillium lilacinum* *

| | Total fatty acids excluding unsaponifiable | | |
|---|---|---|---|
| | *A. nidulans* | | *P. lilac* |
| Fatty acid | %, wt./wt. | %, mole | %, wt./wt. |
| Myristic | 0.7 | 0.8 | 0.1 |
| Palmitic | 20.9 | 22.5 | 32.3 |
| Stearic | 15.9 | 15.5 | 9.4 |
| Arachidic | ... | ... | 1.4 |
| Behenic | 1.4 | 1.3 | |
| Lignoceric | | | |
| Hexadecanoic | 1.2 | 1.3 | 3.4 |
| Oleic | 40.3 | 39.5 | 38.6 |
| Linoleic | 17.0 | 16.8 | 13.4 |
| Linolenic | 0.2 | 0.2 | |
| Unsaturated $C_{20}$ | 2.4 | 2.1 | 1.4 |
| Free acidity, % oleic | 0.8 | ... | 0.2 |

* Compiled from data presented by J. Singh, T. K. Walker, and M. *Biochem. Jour.,* **61**: 85 (1955); and by J. Singh, S. Shah, and T. K. Walker *Jour.,* **62**: 222 (1956).

son with that of other microorganisms (Singh et al., 1955). Th acid content of the fat was a little higher than that of olive oil. of *P. lilacinum* was solid at room temperature and possessed a free acidity (Singh et al., 1956).

**The Fat Content of Molds.** The fat content of different mol considerably, depending in part on the conditions of production

| Organism | of dry pad, g./100 ml. | Sterol, per cent | Lipid, per cent | otein (N × 6.25), per cent | hydrate (by difference), † per cent | fatty acid of lipid (as oleic), per cent | of dry pad, g./100 ml. | Sterol, per cent | Lipid, per cent | protein (N × 6.25), per cent | hydrate (by difference), ‡ per cent | Free fatty acid of lipid (as oleic), per cent |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Aspergillus aerea* 4700A | 2.67 | 0.65 | 4.7 | 34.4 | 60.9 | .... | 2.55 | 0.88 | 6.1 | 25.6 | 68.3 | |
| *A. carbonarius* 4030.1 | 4.04 | 0.39 | 1.1 | 13.7 | 85.2 | 56.3 | 5.03 | 0.33 | 2.1 | 12.5 | 85.4 | |
| *A. cinnamomeus* 3534B | 3.63 | 0.42 | 3.4 | 25.0 | 71.6 | .... | 4.16 | 0.54 | 5.0 | 14.4 | 80.6 | |
| *A. citrosporus* 4301.10 | 3.53 | 0.45 | 3.9 | 32.5 | 63.6 | .... | 2.59 | 0.57 | 1.5 | 25.6 | 72.9 | |
| *A. clavatus* 107 | 2.03 | 0.75 | 7.6 | 35.0 | 57.4 | 20.0 | 2.77 | 0.86 | 16.6 | 22.5 | 60.9 | |
| *A. fischeri* 5041 | 2.62 | 0.90 | 6.4 | 31.2 | 62.4 | 15.2 | 2.40 | 0.84 | 13.4 | 28.1 | 58.5 | 8.3 |
| *A. flavipes* 2 | 2.81 | 0.75 | 7.0 | 36.3 | 57.7 | 8.0 | 2.53 | 0.77 | 23.0 | 21.2 | 55.8 | |
| *A. fumigatus* 2 | 3.13 | 0.82 | 3.1 | 31.2 | 65.7 | 51.5 | 2.63 | 0.26 | 1.8 | 24.4 | 73.8 | 73.1 |
| *A. fuscus* 3534c | 2.81 | 0.93 | 3.0 | 28.1 | 68.9 | 30.2 | 3.94 | 0.48 | 5.7 | 18.1 | 76.2 | 16.9 |
| *A. insuetus* 4658.245 | 2.52 | 1.10 | 13.5 | 23.7 | 62.8 | 13.4 | 2.32 | 1.05 | 24.4 | 21.9 | 53.7 | 15.0 |
| *A. lutea* 4640.473 | 2.62 | 0.65 | 3.8 | 36.7 | 59.5 | .... | 2.41 | 0.45 | 4.8 | 26.2 | 69.0 | |
| *A. melleus* 4291.6 | 1.98 | 0.88 | 3.1 | 40.6 | 56.3 | 44.9 | 2.55 | 0.95 | 3.8 | 23.7 | 72.5 | 36.2 |
| *A. minutus* 4894.2 | 2.33 | 0.52 | 7.4 | 32.5 | 60.1 | 27.4 | 2.56 | 1.10 | 18.3 | 23.7 | 58.0 | 10.6 |
| *A. nidulans* 1 | 3.45 | 0.55 | 19.9 | 25.6 | 54.5 | 9.7 | 4.16 | 0.38 | 16.8 | 13.1 | 70.1 | 7.9 |
| *A. niger* 2 | 3.14 | 0.80 | 2.6 | 28.1 | 69.3 | .... | 3.87 | 0.43 | 2.8 | 18.1 | 79.1 | |
| *A. ochraceous* 4700A | 2.21 | 0.61 | 4.5 | 43.1 | 52.4 | 19.7 | 2.98 | 0.44 | 5.2 | 21.9 | 72.9 | 24.3 |
| *A. oryzae* 965 | 1.17 | 0.84 | 5.0 | 33.7 | 61.3 | 19.5 | 1.45 | 1.09 | 5.6 | 36.3 | 58.1 | 19.9 |
| *A. schiemanni* 3534c | 2.39 | 0.78 | 4.1 | 32.5 | 63.4 | 34.2 | 3.47 | 0.81 | 5.2 | 18.1 | 76.7 | 38.5 |
| *A. sydowi* 1 | 2.02 | 1.09 | 5.0 | 26.9 | 68.1 | 8.4 | 2.53 | 0.86 | 5.2 | 20.6 | 74.2 | 8.0 |
| *Penicillium aurantio-brunneum* 4733.5 | 1.72 | 0.87 | 11.8 | 23.7 | 64.5 | 17.6 | 2.21 | 0.77 | 12.9 | 20.6 | 66.5 | 10.7 |
| *P. chrysogenum* 4733.33 | 1.66 | 0.91 | 2.3 | 43.7 | 54.0 | 67.8 | 1.87 | 1.16 | 7.1 | 15.0 | 77.9 | 30.4 |
| *P. cyaneum* 4640.422 | 0.75 | 0.59 | 2.4 | 33.1 | 64.5 | .... | 1.82 | 0.70 | 4.4 | 20.6 | 75.0 | |
| *P. cyaneo-fulvum* 4733.47 | 1.06 | 0.40 | 3.2 | 36.3 | 60.5 | 39.4 | 1.71 | 0.38 | 8.2 | 21.9 | 69.9 | 41.2 |
| *Paecilomyces varioti* 1 | 1.64 | 1.25 | 14.9 | 30.0 | 55.1 | 14.8 | 1.48 | 1.70 | 10.1 | 26.9 | 63.0 | 27.4 |
| Average | 2.41 | 0.75 | 6.0 | 31.6 | 62.5 | 27.6 | 2.92 | 0.78 | 8.8 | 22.5 | 69.6 | 26.2 |

* L. M. Pruess, E. C. Eichinger, and W. H. Peterson, *Zentr. Bakt. Parasitenk.*, Abt. II, **89**: 370 (1934).

† Includes ash; this varies from 2.5 to 8.5 per cent.

‡ Includes ash; this varies from 1.0 to 4.5 per cent.

basis of the dry weight, the mycelium may contain from as littl
cent to as much as about 50 per cent of fat. Mold spores h
reported to contain from 1 to 14 per cent fat (Pruess et al., 1934

Pruess, Eichinger, and Peterson cultivated 24 molds on two
types of media: (1) a glucose–inorganic salts medium containin
carbonate in excess and (2) a glucose–malt-sprouts medium.
the analyses of the dried mycelia of the molds are shown in T
The lipid content of the mycelia varied from 1.1 to 19.9 per cent
average of 6.0 per cent, when the molds were grown on the gluc
ganic salts (synthetic) medium; and from 1.5 to 24.4 per cent,
average of 8.8 per cent, when the molds were grown on the gluco
sprouts (organic) medium. The average lipid content of all t
was over 46 per cent greater when grown on the organic medi
when grown on the synthetic medium. However, some molds, for
*A. nidulans* 1 and *Paecilomyces variota* 1, yielded more lipid on t
medium than on the former medium.

Ward and his associates extracted the crude fat from 61 differen
39 penicillia and 22 aspergilli, using ethyl ether. Of these molds,
tained more than 15 per cent, but only 6 more than 20 per cent
fat, as indicated in Table 188.

Table 188. Some Molds Containing More Than 15 Per Cent of Crude

| Mold | Crude Fat in Dried Mycelium<br>Per Cent |
|---|---|
| *Penicillium bialowiezense* | 17.0 |
| *P. citrinum* Thom | 18.1 |
| *P. hirsutum* Dierckx | 18.4 |
| *P. soppi* Zal | 20.2 |
| *P. javanicum* van Beijma | 22.2 |
| *P. roqueforti* Thom | 22.9 |
| *P. oxalicum* Currie and Thom | 24.4 |
| *P. piscarum* Westling | 26–28 |
| *P. flavocinerium* Biourge | 28.5 |
| *Aspergillus flavus* Thom and Church | 16.0 |

* G. E. Ward, L. B. Lockwood, O. E. May, and H. T. Herrick, *Ind. Eng.*
**27**: 318 (1935). Copyright 1935 by the American Chemical Society and repri
permission of the copyright owners.

Jennison et al. (1957) found that the fat content of 17 represe
species of wood-rot fungi varied from 2.3 to 12.9 per cent (on a dry
when grown with forced aeration in a 1 per cent malt extract so
The average fat content was 7 per cent.

## FACTORS AFFECTING THE FAT CONTENT OF MOLDS

The conditions under which a mold is cultured exert an influence
fat content. The kind of sugar makes a large difference. Gluc
superior to sucrose for the production of fat from *Aspergillus ni*
*Penicillium spinulosum,* and *P. javanicum* (Gad and Walker.
Xylose is also a good carbon source for these molds (Garrido and W

6). Maltose, arabinose, galactose, lactose, and starch are inferior rces of carbohydrate.

itrogen likewise exerts a strong influence on the amount of fat formed. riao and Walker (1956) studied ammonium nitrate, sodium nitrate, ı, ammonium sulfate, and ammonium chloride as nitrogen sources for same three molds. Ammonium nitrate was the best source for all three ds, followed by sodium nitrate (for *A. nidulans* only).

he effects of different concentrations of magnesium sulfate and sodium drogen phosphate were studied by Garrido et al. (1953), Garrido and ker (1953), and Gad and Walker (1954). The influence of potassium determined by the last two groups. These compounds exert marked ts at different concentrations. Some data relating to the effect of esium sulfate and sodium dihydrogen phosphate on fat formation iven in Table 189.

189. Conditions under Which Highest Yields of Fat Were Obtained from Media Containing Sucrose *

| Mold | Basal medium † with addition of | | Fat | |
|---|---|---|---|---|
| | $NaH_2PO_4 \cdot 2H_2O$, g./100 ml. | $MgSO_4 \cdot 7H_2O$, g./100 ml. | G./100 g. of sucrose utilized | G./100 g. of dry felt |
| *llus nidulans* | 0.730 | 0.500 | 13.2 | 41.9 |
| *ium javanicum* | 0.073 | 0.050 | 7.9 | 28.0 |
| *ulosum* | 0.036 | 0.037 | 5.1 | 20.5 |

ared from data presented by A. M. Gad and T. K. Walker, *Jour. Sci. Food* ). 7, 1954, pp. 339–343.

basal medium contained in g. per 100 ml., $NH_4NO_3$, 0.3; $K_2SO_4$, 0.022; $I_2O$, 0.016; and $ZnSO_4 \cdot 7H_2O$, 0.005. Each 100-ml. conical flask contained medium. The initial sucrose concentration was 4.25 g. per flask. The incubae at 25°C. was 8 days for *A. nidulans* and 9 days for each of the other two

-steep liquor increases the yield of fat under certain conditions o and Walker, 1953; Gad and Walker, 1954; Murray and Walker, However, its use may actually decrease the yield in some cases.

**roduction by P. javanicum van Beijma.**[1] This mold was selected ly since it produced the heaviest mats, yielding a considerable of lipid. The mold was described by Beijma.

nold was cultivated in 200-ml. Pyrex Erlenmeyer flasks, into each ı were placed 75 ml. of the following medium:

| | G./Liter |
|---|---|
| Glucose | 200 |
| $NH_4NO_3$ | 2.25 |
| $KH_2PO_4$ | 0.3 |
| $MgSO_4 \cdot 7H_2O$ | 0.25 |

---

ockwood, G. E. Ward, O. E. May, H. T. Herrick, and H. T. O'Neill, *Zentr. sitenk.*, Abt II, **90**: 411 (1934).

A chemically pure glucose (anhydrous) was used when th the ions of various salts was ascertained; otherwise a commer of glucose containing 91.5 per cent of pure glucose, 8 per cent 0.4 per cent dextrin was used.

The pH of the medium was 4 to 5; the temperature of incubati and the period of incubation 12 days.

In Table 190 is shown the effect of varying the glucose conc

TABLE 190. THE EFFECT ON FAT PRODUCTION OF VARYING THE GLUC CONCENTRATION *

| Glucose, per cent | Mat weight, per cent | Fat, per cent | Culture acid, equivalent ml. $N/10$ acid | Glu su |
|---|---|---|---|---|
| 20 | 2.522 | 29.0 | 149 | |
| 30 | 2.400 | 34.6 | 209 | |
| 40 | 1.964 | 41.5 | 148 | |
| 50 | 1.021 | 35.2 | 20 | |

* L. B. Lockwood, G. E. Ward, O. E. May, H. T. Herrick, and H. T. O'N *Bakt. Parasitenk.*, Abt. II, **90**: 411 (1934).

The greatest mat weight occurred when the glucose concentra 20 per cent, the greatest titrable acidity at 30 per cent, and the percentage of fat at 40 per cent.

Xylose, galactose, maltose, sucrose, glycerol, starch, and dext lipids, xylose being a particularly good source of carbon.

*P. javanicum* utilized sodium, potassium, magnesium, or nitrates; sodium nitrite; ammonium chloride or ammonium su sources of nitrogen; but ammonium nitrate was the best source. nium chloride and ammonium sulfate caused the pH to drop too

The growth and metabolism of *P. javanicum* were favored by t ence of 2.25 to 3.375 g. of $NH_4NO_3$, 0.3 to 1.2 g. of $KH_2PO_4$, an 0.25 g. of $MgSO_4 \cdot 7H_2O$ per liter of medium.

Chromic, columbic, ferric, molybdic, and tungstic ions sti mycelial growth, fat and citric acid formation, and glucose consu

The pH range of 3.1 to 6.8 was favorable, the final pH of the being about 2.5 in each case.

Increasing the air pressure within the culture flask tended to the metabolism of *P. javanicum.*

Decreasing the ratio of surface area to volume decreased the e of the conversion of glucose to lipids.

The production of fat from glucose by *P. javanicum* in a lar laboratory apparatus is described by Ward, Lockwood, May, and in a later report.

The composition of two media used for growing molds for lipid tion, known as "G solution" and "M solution," of which M soluti the more consistent results, is shown in Table 191.

TABLE 191. SOME MEDIA USED FOR GROWING MOLDS FOR LIPID PRODUCTION

| G solution | | M solution | |
|---|---|---|---|
| Substance | G./liter | Substance | G./liter |
| nercial glucose | 220 | Commercial glucose | 220 |
| $O_3$ | 4.50 | $NH_4NO_3$ | 2.25 |
| | 0.40 | $KH_2PO_4$ | 0.3 |
| | 0.216 | $MgSO_4 \cdot 7H_2O$ | 0.25 |
| $\cdot 7H_2O$ | 2.00 | | |

**t Production by A. fischeri.** Prill, Wenck, and Peterson (1935) studied the effect of various factors, such as glucose concentration, nium nitrate concentration, acidity, alkalinity, temperature, aera- ind incubation period, on fat production by *A. fischeri.*

mold was cultured at 30°C. in 500-ml. Pyrex Erlenmeyer flasks, of which contained 100 ml. of the following solution, except where ture of the experiment required otherwise.

| | G. |
|---|---|
| Commercial glucose (cerelose) | 20 |
| $NH_4NO_3$ | 1.00 |
| $KH_2PO_4$ | 0.68 |
| $MgSO_4 \cdot 7H_2O$ | 0.50 |
| $FeCl_3 \cdot 6H_2O$ | 0.016 |
| $ZnSO_4 \cdot 7H_2O$ | 0.005 |
| Distilled water, to make 100.0 ml. | |
| $CaCO_3$ | |

mold growth was destroyed at the end of the incubation period oclaving it at 120°C. for 10 min. Mycelial mats were then washed ater, dried for 2 days at 37°C., ground, and extracted with hot e alcohol for 12 hr. or more.

h fat content in the mycelium was favored by a high concentration ose, a low concentration of ammonium nitrate ($NH_4NO_3$), and a or slightly alkaline solution. (Compare with fat production by *poron pullulans.*) For further details consult the paper by Prill, and Peterson.

**Production by Fusarium Species.** The role of *Fusarium* species ormation has been reviewed by Hesse (1949).

n (1943) described the submerged-culture production of fat, using *Fusarium* species. The mold was grown in a medium containing nt carbohydrate; a nitrogen source, such as ammonium salts, urea, amino acids, or acid amides; a phosphorus source; and an t supply of potassium and magnesium. The fermentation was out at a temperature of 24 to 27°C., at a pH of 2 to 4 (to avoid of the carbohydrate and formation of alcohol), and with aera- t formation was optimal at 40 to 48 hr. The fat constituted nt of the dried weight of the mycelium. Twelve to 15 kg. of dry as obtained per cubic meter.

**Fat Production by Geotrichum (Oïdium, Oospora).** In 1926, Chapman isolated a species of *Geotrichum* from a sewer bl the growth of this organism. Culture of this species in a nutrient led to the formation of a thick film within 2 days, which containe cent fat and 50 per cent protein. The flavor and odor of the film re cream cheese.

Out of 50 strains of *G. candidum* examined—10 of which produc siderable quantities of fat—Fink, Haeseler, and Schmidt (1937) two, strains A and B, which reproduced well and produced good y fat. Of these two strains, strain A was preferred, for it was less s to higher temperatures and it reproduced more uniformly.

*G. candidum,* or, as it has been commonly designated in th *Oïdium lactis,* is frequently found in Camembert cheese and i butters and may be a cause of spoilage of cream cheeses. It i resistant to heat and infection by other microorganisms. This at of growing in a medium that has become infected by other microorg and, at the same time, giving good results makes the mold esp desirable.

*Optimum Conditions for Fat Production.* Since *G. candidum* naturally in dairy products, whey is an excellent source of raw ma Whey may be used alone or enriched with other nutrient subst Urea, ammonium sulfate, and ammonium acetate are good sour nitrogen, while primary potassium phosphate ($KH_2PO_4$) and magn sulfate are excellent sources of potassium, phosphate, magnesium sulfur.

As an illustration of the nature of the nutrient medium and the r obtained when *G. candidum* is grown in Jena flasks, the following e ment is quoted: The nutrient solution contained 2 liters of whey, 2 ammonium sulfate, 1 g. of primary potassium phosphate, and 0.5 magnesium sulfate. In each of a series of Jena flasks, 250-ml. po were placed. Data for the experiment are shown in Table 192.

TABLE 192. DATA ON FAT PRODUCTION BY *Geotrichum candidum* *

| Age, days | Yield in dry material, g. | Nitrogen, per cent of dry substance | Crude protein extraction, per cent of dry substance | Crude fat, per cent of dry substance | Total yield of crude fat, g. | pH nu sol |
|---|---|---|---|---|---|---|
| 2 | 1.777 | 5.40 | 33.75 | 7.5 | 0.133 | |
| 3 | 3.238 | 3.32 | 20.75 | 10.8 | 0.349 | ( |
| 4 | 3.968 | 3.15 | 19.69 | 13.4 | 0.531 | |
| 5 | 4.771 | 2.98 | 18.62 | 16.7 | 0.796 | |
| 6 | 5.729 | 2.81 | 17.56 | 22.5 | 1.290 | |
| 7 | 6.147 | 2.76 | 17.25 | 22.0 | 1.352 | |
| 12 | 5.886 | 2.85 | 17.81 | 19.6 | 1.152 | |
| 16 | 5.710 | 3.15 | 16.69 | 16.6 | 0.948 | |

* H. H. Fink, H. Haehn, and W. Hoerburger, *Chem. Ztg.*, **61**: 744–747 (1937).

examination of this table shows that under the conditions of the
iment the maximum yield of crude fat was obtained in 6 days.
her experiments by Fink, Haeseler, and Schmidt have indicated that
allow pan or dish was superior to the Jena flask as a container.
e optimum temperature for fat production was 25 to 30°C.
sugar concentration of 4 to 6 per cent was satisfactory for both
s A and B.

ng strain A, Fink and his coworkers obtained yields of 12.5 to
g. of crude fat within 5 days from 100 g. of sugar in whey enriched
ammonium sulfate, potassium chloride, and magnesium sulfate.
yields, according to Fink and his associates, were better than those
ed by Geffers (1937) from pure wheys, calculated on the sugar
d, when using strains of *G. wallroth*, strains similar to *G. candidum*,
ere secured in a shorter period of time. However, Geffers obtained
as high as 50 per cent of the dry weight of *G. wallroth*, when
actose.

*luction from Straw and Oat Hulls.* A German process for the pro-
n of fat by *G. candidum* from the sugars derived from straw (by a
ed Scholler process) and from oat hulls has been described by Balls.
ypes of apparatus were used. One of these consisted of a rotating
made by stretching canvas over a wooden frame about 4 ft. in
er, which was open at the ends near the central shaft. The nutrient
n was placed on the inside of the drum. In order to inoculate the
n, the inside surface of the canvas was sprayed or painted with a
of *G. candidum*. The drum was rotated about two times a minute
operation. The second type of apparatus consisted of a row of
of canvas hung about 3 in. apart on a wooden frame. One side of
vas was painted with a culture of the organism. Nutrient solution
mped through nozzles onto the other side of the canvas and
down to a tray below. It was pumped continuously from the trays
anvas until the sugar was nearly consumed. Filtered air was cir-
between the canvas sheets. The process was complete in about
at room temperature. The yield was said to be about 20 per cent
a dry basis. For further details, the reader is referred to Balls's

## References on Production of Fat by Molds

K.: *FIAT Final Report No.* 371, Oct. 10, 1945.
. H.: The Production of Fat by a Species of *Penicillium* Grown in Sucrose
ion, *Jour. Soc. Chem. Ind. London,* **46**: 200T (1927).
roduction of Fat from Carbohydrates and Similar Media by a Species of
*llium, Biochem. Jour.,* **33**: 1158 (1929).
oe Kingma, F. H. van: Mykologische Untersuchungen-*Penicillium javani-*
ov. spec., *Verhandel. Akad. Wetenschap. Amsterdam, Afdeel. Natuurk.,*
16 (1929).
*Bull. soc. chim. biol.,* **8**: 1081 (1926).
. A., Jr.: The Fermentation of Sugar-cane Products, *Jour. Am. Chem. Soc.,*
5 (1906).
: Eine neue biochemische Fettsynthese, *Chem. Ztg.,* **5**(6): 47 (1943).
., H. Haehn, und W. Hoerburger: Über die Versuche zur Fettgewinnung

mittels Mikroorganismen mit besonderer Berücksichtigung der A Instituts für Gärungsgewerbe, *Chem. Ztg.,* **61**: 689–693; 723–726; 744- (A very comprehensive article with 52 references on fat production organisms.)

———, G. Haeseler, und M. Schmidt: *Zeit. Spiritusind.,* **60**: 74, 76, 81 (19

Gad, A. M., and T. K. Walker: I. Media Conducive to Formation o Sucrose by *Aspergillus nidulans, Penicillium javanicum* and *Penicilli sum, Jour. Sci. Food Agr.,* No. 7, 1954, pp. 339–343.

Garrido, J. M., y T. K. Walker: Determinación de las concentraciones sulfato magnésico, fosfato sódico y sulfato potásico para la máxima de glucosa en grasa por *Aspergillus nidulans* Eidam, *Penicillium ja* Beyma y *Penicillium spinulosum, Anales real soc. españ. fís. y qui* **49**: 839–846 (1953).

——— and ———: II. Synthesis of Fat from Various Carbohydrates in S tures of *Aspergillus nidulans, Penicillium javanicum* and *Penicillium* and the Influence of the Nitrogen Source on the Synthesis of Fat fro *Jour. Sci. Food Agr.,* No. 4, 1956, pp. 233–237.

———, M. Woodbine, y T. K. Walker: Estudio de la acción de diversas ciones de magnesio, fósforo y potasio, sobre la formación de grasa por *nidulans* Eidam, *Penicillium javanicum* V. Beyma y *Penicillium s Anales real soc. españ. fís. y quim. Madrid,* **49**: 829–838 (1953).

Geffers, H.: *Arch. Mikrobiol.,* **8**: 66–98 (1937).

Hesse, A.: Industrial Biosyntheses. Part I. Fats, *Advances in Enzymolog* 704 (1949).

Jennison, M. W., C. G. Richberg, and A. E. Krikszens: Physiology of Wo Basidiomycetes. II. Nutritive Composition of Mycelium Grown in S Culture, *Appl. Microbiol.,* **5**(2): 87–95 (1957).

Kroeker, E. H., F. M. Strong, and W. H. Peterson: The Chemistry of Mol VII. The Lipids of *Penicillium aurantio-brunneum, Jour. Am. Chem.* 354 (1935).

Lockwood, L. B., G. E. Ward, O. E. May, H. T. Herrick, and H. T. O'N Production of Fat by *Penicillium javanicum* van Beijma, *Zentr. Bak tenk.,* Abt. II, **90**: 411 (1934).

Murray, S., and T. K. Walker: III. Media Conducive to Formation of Sucrose by *Penicillium soppi* Zaleski in Surface Culture, *Jour. Sci. F* No. 4, 1956, pp. 237–240.

Pearson, L. K., and H. S. Raper: The Influence of Temperature on the Fat Formed by Living Organisms, *Biochem. Jour.,* **21**: 875 (1927).

Peterson, W. H., L. M. Pruess, H. J. Gorcia, and H. C. Greene: Large-scale tory Cultivation of Molds, *Ind. Eng. Chem.,* **25**: 213 (1933).

Pontillon, C.: Contribution à l'étude physiologique des lipides du *Sterigm nigra, Rev. gén. botan.,* **44**: 465, 526 (1932); **45**: 20 (1933).

Prill, E. A., P. R. Wenck, and W. H. Peterson: The Chemistry of Moul VI. Factors Influencing the Amount and Nature of Fat Produced by *A fischeri, Biochem. Jour.,* **29**: 21 (1935).

Pruess, L. M., E. C. Eichinger, and W. H. Peterson: The Chemistry of Mol III. Composition of Certain Molds with Special Reference to the Lipid *Zentr. Bakt. Parasitenk.,* Abt. II, **89**: 370 (1934).

Rockwell, G. E., and F. O'Flaherty: Studies in the Physiology of Moulds. position and Culture of Moulds. *Jour. Am. Leather Chemists' Assoc.,* (1931).

Singh, J., S. Shah, and T. K. Walker: The Component Fatty Acids of *Pe lilacinum* Fat, *Biochem. Jour.,* **62**: 222–224 (1956).

——— and T. K. Walker: Changes in the Composition of the Fat of *As nidulans* with Age of Culture, *Biochem. Jour.,* **62**: 286–289 (1956).

———, T. K. Walker, and M. L. Meara: The Component Fatty Acids of th *Aspergillus nidulans, Biochem. Jour.,* **61**: 85–88 (1955).

Strong, F. M., and W. H. Peterson: Chemistry of Mold Tissue. IV. The *Aspergillus Sydowi, Jour. Am. Chem. Soc.,* **56**: 952: 1934.

.an. M. X.: Some Organic Constituents of the Culture Solution and the Myce-
num of Molds from Soil, *Science,* **38**: 678 (1913).

une, E. F., and R. Bonnet: L'energie de croissance X.—Formation des matières
rasses au dépens de glucides chez les microorganisms, *Bull. soc. chim. biol.,* **9**:
88 (1927).

. G. E., and G. S. Jamieson: The Chemical Composition of the Fat Produced by
*enicillium javanicum* van Beijma, *Jour. Am. Chem. Soc.,* **56**: 973 (1934).

-, L. B. Lockwood, O. E. May, and H. T. Herrick: Production of Fat from
lucose by Molds. Cultivation of *Penicillium javanicum* van Beijma in Large-
ale Laboratory Apparatus, *Ind. Eng. Chem.,* **27**: 318 (1935).

ey, D. W., F. M. Strong, W. H. Peterson, and E. A. Prill: The Chemistry of
old Tissue. X. The Phospholipids of *Aspergillus Sydowi, Jour. Am. Chem.
oc.,* **57**: 2589 (1935).

## CHAPTER 41

# MOLD ENZYME PREPARATIONS: USES AND PR

## MOLD ENZYMES

Mold enzymes exercise extremely important roles in our wa
They are important in the production of fermented food items
cheeses and soy sauce; organic acids, such as citric and gluc
antibiotics, such as penicillin and fumagillin. They are useful
forming proteins, carbohydrates, and fats, under controlled cond
simpler compounds. They find large use in saccharifying starche
production of alcohol and other valuable end products. They
important in bringing about undesirable changes, such as thos
ated with plant diseases, food spoilage, textile deterioration, a
decay.

Mold enzymes may be produced by a variety of methods; for
they may be produced in tanks under submerged-culture conditi
manner similar to that used for the production of antibiotics, th
be produced by growing the mold on shallow layers of substrate
or they may be produced in rotating drums.

**Submerged-culture Method.** Data relating to the submerged
production of amylolytic enzymes by molds have been presented i
cations of Van Lanen and Le Mense (1946), Le Mense et al.
Corman and Langlykke (1948), the U.S. Department of Agr
(1950), Shu and Blackwood (1951), Pool and Underkofler (1953),
kofler and Hickey (1954), Forbath (1957), and others. One of the
manufacturers of microbial enzymes in the United States produc
50 per cent of its output by the submerged-fermentation process (F
1957).

In the submerged-culture process, the raw materials are mix
tank; heated, cooked, and sterilized by a batch or continuous
cooled to the temperature optimum for the growth of the mold;
or allowed to flow by gravity to a fermentor; and seeded with the
mold. As the microbe grows under aerobic conditions, it produ
enzymes. At the time of maximum enzymatic activity, the mold
lium is removed by filtration, and the enzymes are separated fr
liquor by solvent or salt precipitation and further processed. The
enzyme-containing liquor may be used directly in some cases.

**Continuous-tray Method for Producing Mold Enzymes.** J
(1948) has described a method for producing mold enzymes by
tinuous-tray method. He states that it eliminates considerable

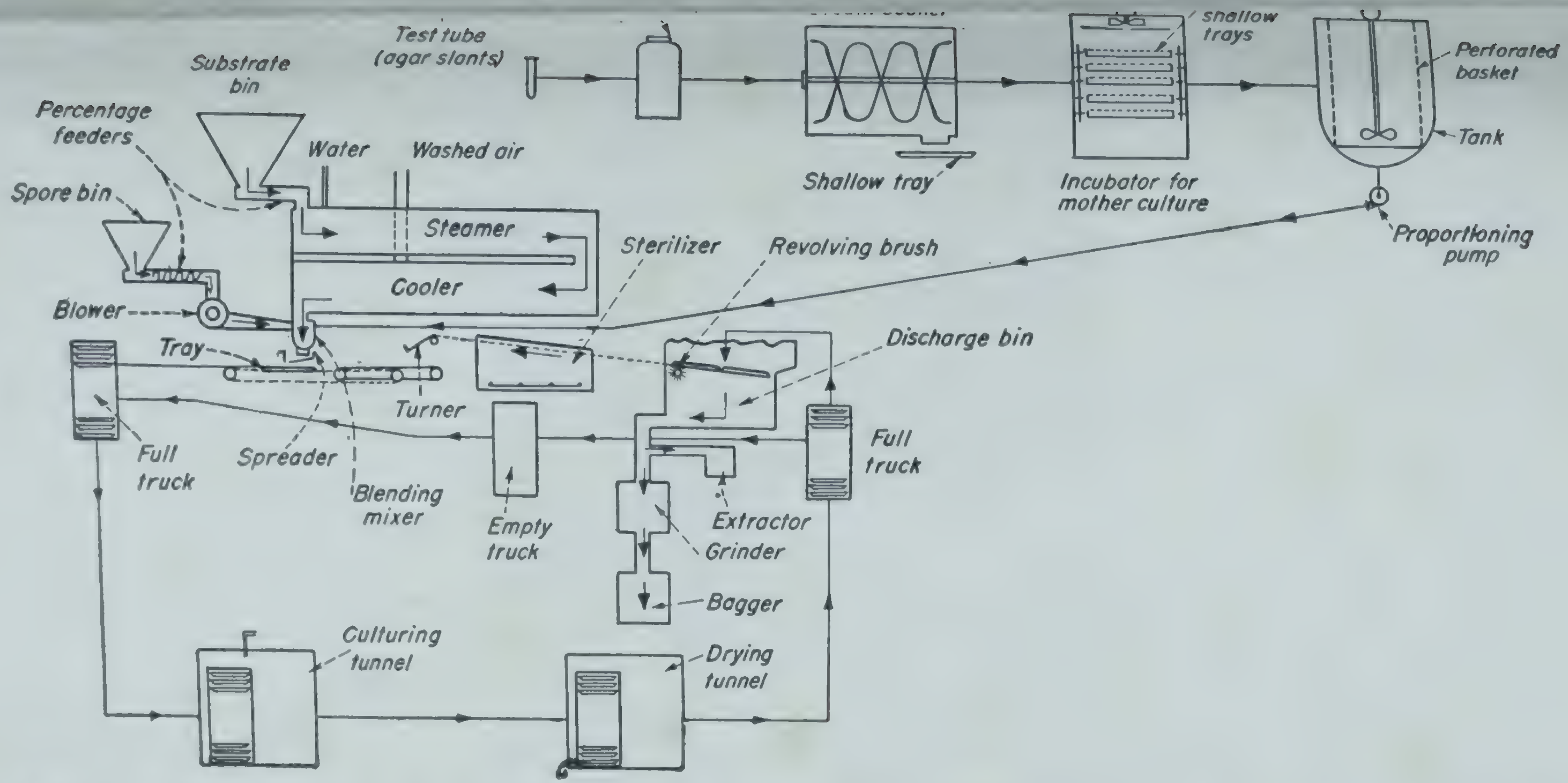

Test tube
(agar slants)
shallow
trays
Perforated
basket
Substrate
bin
Percentage
feeders
Water
Washed air
Tank
Spore bin
Steamer
Shallow tray
Incubator for
mother culture
Proportioning
pump
Sterilizer
Revolving brush
Cooler
Blower
Discharge bin
Tray
Turner
Full
truck
Spreader
Full
truck
Blending
mixer
Empty
truck
Extractor
Grinder
Bagger
Culturing
tunnel
Drying
tunnel

FIG. 112. Diagrams of process for producing mold enzymes by tray method. [*Courtesy of G. A. Jeffreys, Food Inds.*, 20: 688 (1948).]

labor normally required for handling trays in older processes. T consists of preparing an inoculum, sterilizing the substrate and inoculating the substrate, placing the inoculated substrate in tr ing the trays on trucks, incubating the trays in special incubat the mold product in tunnels, and grinding the dried product an it. A diagram of the process is shown in Fig. 112.

*Preparation of the Inoculum.* The mold *Aspergillus oryzae* on suitable agar slants. These cultures are used to inoculate moist bran medium in 2-qt. jars. After adequate sporulation place, the contents of a jar may be used to inoculate 100 lb. of and cooled bran or mother culture. The latter is grown in designed shallow trays (Fig. 113) equipped with perforated

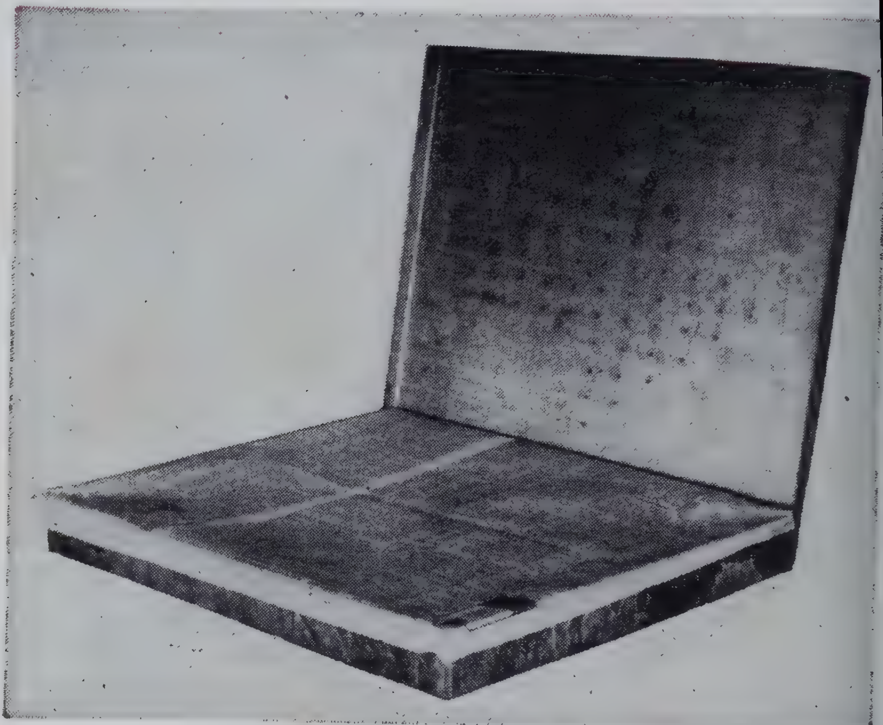


Fig. 113. Culture tray. [*Courtesy of G. A. Jeffreys, Food Inds.,* **20**: 688 (

piece of newspaper is placed over the top of the tray before the put on, which, according to Jeffreys, prevents contamination and moisture. The trays containing the mother cultures are placed o in special incubator rooms in which the temperature is maintai approximately 78°F. by means of forced-draft circulation and heati cooling coils. After a suitable incubation period, the mold-spore pr tion is ready for drying or for inoculation purposes. The tray cov removed to facilitate drying of the mother cultures.

*Preparation and Inoculation of Substrate.* The substrate is fed conveyor by a percentage feeder, where it is mixed with the re amount of water. The mixture is heated to 190°F., or higher, with and maintained at this temperature for approximately 15 min steamed mixture is dropped onto a conveyor, where cooling is b about by a combination of cold, filtered air and a water jacket.

loist and dried spores are used to inoculate the prepared substrate, inoculum amounting to about 0.5 per cent on a dry basis. The moist es are prepared by transferring the undried mother culture to a perfo- d basket in a tank and suspending the spores in sterile water, using nanical agitation. By means of a proportioning pump the spore sus- ion is forced to a blending mixer, where it is sprayed upon the sub- e. At the same time, the substrate is inoculated with dry spores, h are fed in from a bin by means of a percentage feeder and a blower. substrate and spores are thoroughly mixed together.

*aying and Incubation.* The inoculated substrate is dropped onto a ider, which distributes it uniformly in sterilized trays. The loaded are conveyed to a loading point and loaded onto a truck, the latter ng 20 trays, 4 in. apart. The trucks are pushed into culturing tunnels ial incubators) on overhead trolleys. Air is circulated in the cultur- unnels by means of fans located 6 ft. apart between the ceiling and iling which draw in the air, fresh or recirculated (refer to Fig. 114). ring incubation the temperature of the substrate is kept from rising 37°F. by circulating cool, humidified air through the culturing ls. When it becomes necessary to warm the air of the tunnels, it is nplished by the use of "thermostatically controlled steam jets."

*ying and Bagging.* After growth on the substrate has reached an um, usually after 24 to 30 hr., the trucks with their contents are orted into the drying tunnels, which consist of primary and second- ages with a center exhaust. The air flows parallel to the direction movement of the trucks in the first stage and countercurrent to lirection in the secondary stage. The temperature of the enzyme- ning product is not allowed to exceed 110°F. during the drying s, wherein the moisture content is reduced to about 8 per cent. dried material is ground and bagged, or it may be extracted.

*dling of Trays.* After the contents of the trays are removed, they ried on a chain belt in the inverted position over revolving brushes clean them. They are next conveyed through an oven, where they jected to a temperature of 300°F. for 3 min. After inversion and ;, they are ready for reloading.

**ating Drum Method for Producing Mold Enzymes.** A method ducing amylases by the use of rotating drums with controlled aera- d temperature has been described by Underkofler and associates (refer to page 846). Products made by this method were high vity.

**ose Oxidase.** This enzyme is a flavoprotein or yellow enzyme and ical with the antibiotic notatin. The enzyme may be produced by *ium chrysogenum, P. notatum,* or *P. resticulosum* (Brian, 1951). presence of glucose and oxygen, it is strongly bactericidal, owing roduction of hydrogen peroxide. It is thermolabile.

se oxidase may be used for removing glucose or oxygen from food ls. It is used by egg driers for removing glucose from egg whites, e eggs and yolks, according to the following reaction:

Glucose + Oxygen + Glucose oxidase → Gluconic acid

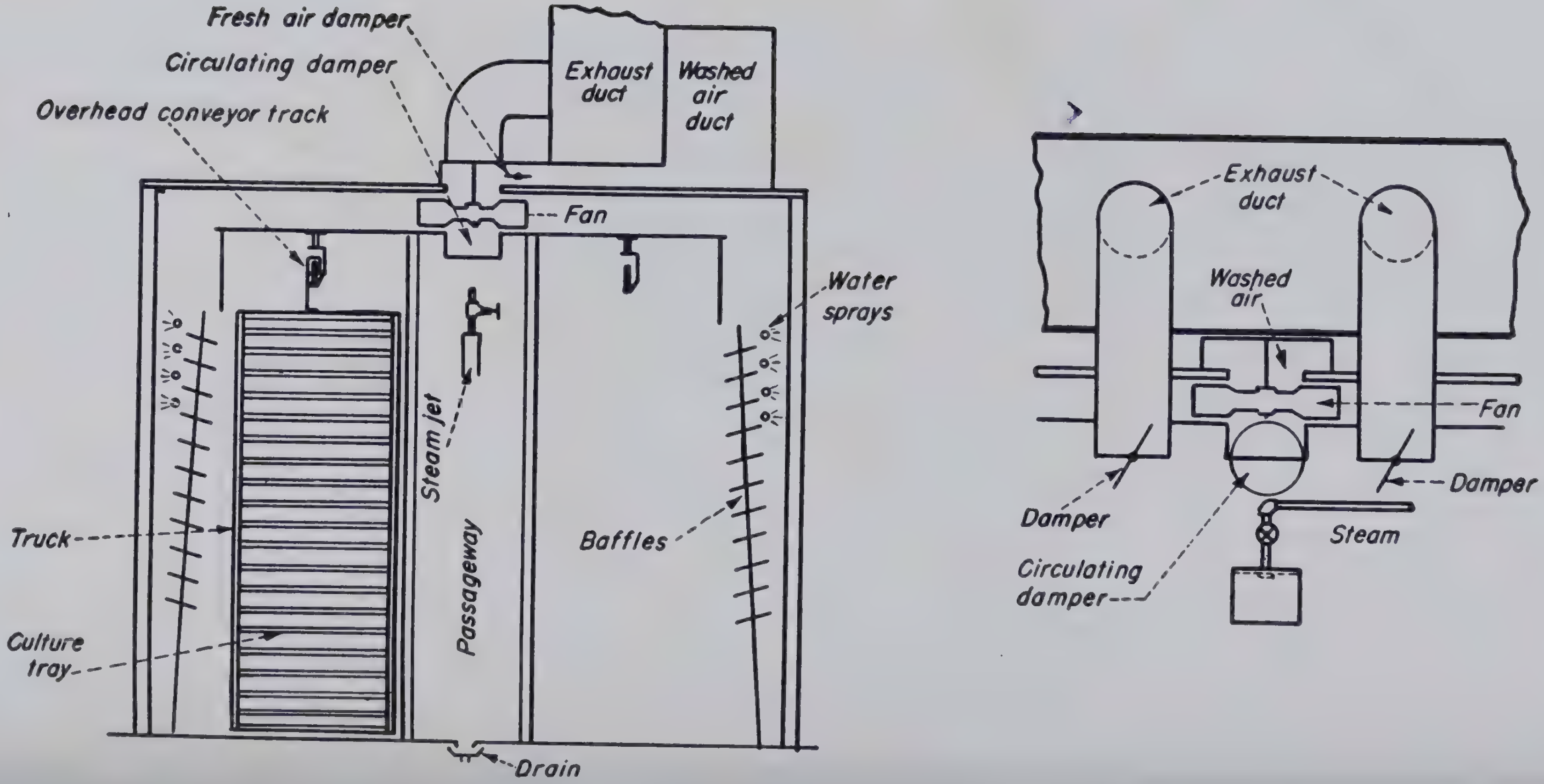

Fresh air damper
Circulating damper
Overhead conveyor track
Exhaust duct
Washed air duct
Fan
Water sprays
Steam jet
Truck
Passageway
Baffles
Culture tray
Drain
Exhaust duct
Washed air
Fan
Damper
Damper
Steam
Circulating damper

Fig. 114. Culturing tunnel. [Courtesy of C. A.

ce an excess of oxygen is required for bringing about this reaction, lrogen peroxide is added as a source of oxygen, and the enzyme catalase upplied to liberate the oxygen as indicated in the following reaction:

$$2H_2O_2 + \text{Catalase} \rightarrow 2H_2O + O_2$$

n one process described by Snyder (1953), the liquid egg whites are ed preferably in a jacketed stainless-steel tank provided with an agi- r capable of rotation at a low rate of speed and a cover to prevent n drying. The pH of the liquid egg whites is adjusted to 7.0 to 7.5 hydrochloric, lactic, or citric acid. The temperature of the mix is ght to 85 to 90°F. and held at this level during the treatment. Usu- 50 g. of the concentrated enzyme powders, or 1 lb. of the dilute liquid mes, is added for the treatment of each 1,000 lb. of liquid egg white. ingredients are gently mixed, and agitation is continued during the ment. Hydrogen peroxide of 35 per cent strength is added to supply oxygen necessary for the conversion of glucose to gluconic acid. lly a total of 0.5 per cent, or 5 lb., of hydrogen peroxide is added for lb. of liquid egg white. The addition is best accomplished continu- by means of a proportioning device during the first 6 hr. of the treatment period.

ucose oxidase may be used for removing oxygen from beer or other ned, bottled, or packaged) food materials (Lockwood, 1952). Ordi- y 3 to 4 oz. of the powdered concentrate, or 2 lb. of the dilute liquid ne, is adequate for the treatment of 100 barrels (31 gal.) of beer der, 1953).

cose oxidase and catalase may be used to protect certain foods t rapid oxidative deterioration. In this case, glucose should be t in excess. Wrapping paper may be coated or impregnated with enzymes and their necessary adjuncts and used to protect foods. pplication is covered in U.S. Patent 2,765,233, which was issued to and Scott.

ethod for removing the free oxygen from enclosed spaces has been ed by Scott (1956). The components of the oxadise-catalase e system, together with glucose and appropriate buffers, are pack- n an air-permeable, water-impermeable film, such as, for example, ylene. The deoxygenation packet appears to offer large promise nection with the extension of the storage life of dehydrated, dried, her foods which contain constituents sensitive to oxygen.

**teases from Mortierella renispora.** Wetter (1952, 1954*a*, *b*) l the extracellular proteases isolated from the culture medium of *rella renispora* Dixon-Stewart (PRL 26).

mold was grown in a sterilized medium consisting of 2 per cent kim-milk solids, 2 per cent glucose, 0.1 per cent monobasic potas- osphate, and tap water and contained in a 30-liter stainless-steel or similar to those described by Rivett, Johnson, and Peterson The sugar and skim milk were sterilized separately in some cases e the browning of the medium. Fifteen liters of medium were ed with 600 ml. of a 3-day culture of the mold grown in the same

medium but in shake flasks. The temperature was kept at 30°C
was used, and sterile air was supplied at the rate of 0.5 liter
medium per minute. After about 60 hr., the mycelium was sepa
the fermentation liquor, which contained the enzymes, was stor
under toluene. The specific activity of the protease concentrat
by repeated precipitation with ammonium sulfate compared
with that of crystalline trypsin.

Wetter (1954*a*) separated the protease concentrate of *M. re*
zone electrophoresis into two proteases, each of which hydroly
denatured hemoglobin. The pH optima were similar.

**Location of Enzymes.** Based on the results of studies conce
the inactivation of the enzymatic activity by treatment of the s
0.1 *N* HCl, Mandels (1953) concluded that the invertase of the
*Myrothecium verrucaria* (QM 460) and the invertase, cellob
helase, and maltase of the spores of *A. luchuensis* (QM 873) ar
at the external surface of the plasma membrane.

**Pentosan Breakdown.** Simpson (1954) surveyed microorga
the production of enzymes that possessed the ability to attack t
sans of wheat flour. The more active genera found included *Al*
*Aspergillus, Fusarium, Trichothecium, Trichoderma,* and *Bacillus*

**Crystalline Enzyme from Trichoderma koningi.** Toyama
reported on the preparation of a crystalline enzyme from *T.*
which possessed cellulytic and amylolytic activities. The extrac
mold from wheat-bran and sawdust culture was stated to
very high activities of cellulase, carboxymethylcellulase, cellobi
amylase.

**Cellulase from Myrothecium verrucaria.** The enzyme cellul
verts cellulose to cellobiose. Whitaker (1951; 1952; 1954*a*,
Whitaker and associates (1954) have carried out extensive rese
the cellulase produced by *M. verrucaria*. He showed (1954*b*) that
of $\beta$-1,4′-oligoglucosides may be hydrolyzed by the cellulase of th

**Uses of Mold Enzymes.** New uses for mold and other m
enzymes are being found from time to time. Presently the most im
mold enzymes, based on industrial applications, are (1) those w
upon starches; (2) the proteases, which may be considered to incl
proteinases and the peptidases; (3) glucose oxidase; and (4) t
tinases (pectinesterase and polygalacturonase). Enzymes of lesser
tance are lipase and invertase.

Enzymes which act upon starches, or their breakdown product
possess dextrinizing or liquefying action, usually a special attri
$\alpha$-amylase; limit-dextrinase activity; and glucogenic ability (
activity). The enzymes produced by a certain mold are usuall
ticularly desirable for a given purpose. The enzymes which ac
starches are used in the preparation of sizes and adhesives, in the
ing of textiles, for the removal of starch from apple pomace duri
manufacture of pectin, in the pharmaceutical trade, and for othe
poses. After the desired conversion has taken place, the action
enzymes may be destroyed by the application of heat.

roteases, which may be obtained from members of the *A. flavus-oryzae* ip, *M. renispora*, or bacteria, are used for several purposes. They may ised in the degumming of silk goods, in the unhairing and bating of s, in the manufacture of liquid glue, as a substitute for or combined soap in the laundry business, and as an agent in the ripening of se. In the preparation of liquid glue, proteases partially hydrolyze glue. Proteases are said to be the best agent used for this purpose in reparation of liquid glue, as controlled operations may thus be car- out. Proteases may also be used in making chillproof beer.

ngal enzymes (amylases and proteases) are being used in the baking stry with good results (Conn et al., 1950; Reed, 1952*a*, *b*). Bread ared with the use of such enzymes possesses good crust color and loaf ne, improved grain and texture, and greater softness and uniformity. enzymes concerned act chiefly during the fermentation stage. They nactivated during the baking process at a lower temperature than enzymes. This is an advantage, because it prevents excessive action e oven, which occasionally leads to the formation of sticky crumb. e preparation and uses of glucose oxidase have been referred to dy.

tinesterase and polygalacturonase are used in the clarification of uices. They are also important in retting processes, as in the making en from flax.

further details on the applications of mold enzymes, the reader is ed to the publications of Reed (1950, 1952*a*, *b*), Smythe (1951), on (1953), Underkofler and Hickey (1954), Underkofler and Ferra- 1957), and others.

**eral References.** Detailed information on the enzymes may be in the publications of Bamann and Myrbäck (1941); Lardy (1949); r (1949); Stetter (1951); Sumner and Myrbäck (1951); Haehn ; Sumner and Somers (1953); Colowick and Kaplan (1955); Nei- Stumpf, and Stanier (1955); Mehler (1957); and others; and also *bnisse der Enzymforschung* and in *Advances in Enzymology*.

**ts.** Tables 193 and 194 present information in chart form regard- roorganisms which produce enzymes and applications of enzymes, ively.

## SOME MOLD PRODUCTS

ls have been used for many centuries in such countries as Japan hina on account of the desirable changes which they produce in substances. In the preparation of soybean sauces, sake, and vari- er products, molds, especially of the *Aspergillus flavus-oryzae* assume much importance.

**Sauce.** This is an item made from soybeans, with the use of *shoyu* he strain of *A. flavus-oryzae* used should possess high proteolytic as well as amyloclastic, since there is very little starch in the sed for the fermentations. The *koji* or starter is prepared by inocu- ooked soybeans, usually mixed with ground roasted wheat, with

TABLE 193. SOME ENZYMES AND MICROBES WHICH PRODUCE THEM

| Enzyme | Bacteria producing | Enzyme | Yeast producing | Enzyme | Molds producing |
|---|---|---|---|---|---|
| Amylases | *Bacillus subtilis, B. macerans, B. polymyxa* | Invertase | *Saccharomyces cerevisiae, S. exiguus* | Amylases | *Aspergillus niger, A. oryzae, Rhizopus oryzae* |
| Cytase′ | *B. subtilis* | Lactase | *S. fragilis, Candida pseudotropicalis (Torula cremoris)* | Cellulase | *Myrothecium verrucaria* |
| Dextransucrase | *Leuconostoc mesenteroides* | | | Glucose oxidase | *Penicillium notatum* |
| Diaminopimelic acid decarboxylase | *Aerobacter aerogenes* | Lipase | *C. lipolytica (Mycotorula lipolytica)* | Lipase | *A. luchuensis* |
| Penicillinase | *B. brevis, Actinomyces candidus* | | | Pectinases: Pectinesterase, polygalacturonase | *Byssochlamys fulvo* |
| | | | | Protease | *Mortierella renispora* |

TABLE 194. SOME ENZYMES OF MICROBIAL ORIGIN AND THEIR APPLICATIONS

| Enzyme | Application |
|---|---|
| ylases | Liquefaction and saccharification of starch |
| -Amylase | Reduction of viscosity of chocolate sirups |
| Liquefying | Sizing of paper and textiles |
| Dextrinogenic | Desizing of textiles |
| Saccharifying | Fruit-juice clarification (when turbidity caused by starch) |
| -Amylase | Production of corn sirup |
| Saccharifying | Modification of dough in baking industry; correction of deficiencies in digestive enzymes |
| transucrase | Production of dextran; production of fructose |
| cose oxidase (notatin or yellow enzyme) | Removal of glucose in eggs before drying; removal of oxygen in presence of glucose |
| rtase | Production of soft centers in candy |
| ase | Prevention of sandiness in dairy products (ice cream, processed cheeses, etc.) |
| se | Cheese making, using pasteurized milk |
| ic enzymes (methylesterase, polygalacturonase) | Clarification of fruit juices and wines; preparation of green coffee; acceleration of filtration of fruit products containing pectin |
| illinase | Destruction of antibiotic activity of penicillin |
| olytic enzymes (peptonizing, peptidizing) | Liquefaction and hydrolysis of casein, lactalbumin, gelatin and other proteins; destruction of gelatin sizes; chillproofing of beer; unhairing of soaked hides; removal of stains |

cted strain of *A. flavus-oryzae* and incubating until each bean is d with sporulating mycelium. The *koji* is placed in a concentrated n chloride brine, wherein an enzymic digestion takes place over a eriod of time—from a few months to a few years. Daily agitation ration are advantageous. Yeasts and bacteria aid in bringing about anges in the bean. The mash which results is dark brown in color f thick consistency. The mash is finally pressed, and the juice or is boiled, filtered, and processed. Soy sauce is used in the manufac- f table sauces, or it may be employed directly.

*nese Soy Sauce.* The production of Chinese soy sauce has been bed by Lockwood (1947), of the Northern Regional Research Lab- y, U.S. Department of Agriculture. Lockwood was aided in his h by Pei Sung King, of the National Bureau of Industrial Research, ing, China, while the latter was working as a guest at the tory.

raw materials used in the process consist principally of soybeans naller amounts of rice, coarsely ground parched wheat, and table

There are three types of microorganisms used in the fermen strains of *A. oryzae* (NRRL 1988 and 1989), a yeast (*Zygosac soyae*, or *Hansenula* NRRL Y1096), and a bacterial culture ( *lus delbrueckii* NRRL B445).

In the production of Chinese soy sauce, there are two main first of these is concerned with the production of five culture (two mold, one yeast, one bacterial, and one combined or soy second, with the brine fermentation of cooked soybeans.

PREPARATION OF THE *Kojies.* The medium used for the prep the mold *kojies* is rice that has been cooked until soft in boil drained, divided into 2 portions, and sterilized. One portion o ilized rice is inoculated with *A. oryzae* NRRL 1988, the oth *oryzae* NRRL 1989. The inoculated rice is incubated until it with green mold growth, which requires 3 to 5 days. According wood, only tested strains of *A. oryzae* should be used in orde the chance of producing sauces of poor quality.

The medium for the yeast and bacterial *kojies* is prepared beans. The soybeans are soaked overnight in water and drain water, 1 qt. for each 3 oz. of soybeans, is added, and the soy cooked at a steam pressure of 15 lb. for 1 hr. The broth thus is dispensed into bottles and sterilized at a pressure of 15 lb. fo The cooled broth is inoculated with a suitable culture of *Z. so Hansenula* NRRL Y1096 and incubated at 30 to 35°C. (85 to 9 1 to 4 days, after which time it may be used. A 2-day-old cultur stored in a refrigerator for several weeks before use, according wood. The bacterial *koji* is prepared by growing *L. delbruecki* B445 in another portion of the soybean broth at a temperature 40°C. This culture also may be used when 1 to 4 days old.

Soybeans are the main ingredient used in preparing the Five lb. of them are soaked in 1 gal. of water for 20 hr., after excess water is drained off. The soybeans are cooked for 3 hr. at pressure of 15 lb. Then 2 lb. 3 oz. of coarsely ground parched added to the cooked soybeans and thoroughly mixed therewith. T ture is spread in layers 2 in. deep on suitable wooden or met The four *kojies* (two mold, one yeast, and one bacterial) are mi the soybeans on the trays, after which the trays are stacked manner as to permit good circulation of air and incubated at 3 85°F.) for 4 to 5 days. Under these conditions the soybeans covered with a growth of *A. oryzae* and ready for the ensui fermentation.

Soy sauce of superior quality may be obtained by inoculating beans with the yeast *koji* a day before the cracked parched added. The growth advantage gained by the yeast results in bett and aroma (Lockwood and Smith, 1950–1951).

THE BRINE FERMENTATION. The soy *koji*, prepared as outline is placed in a suitable deep container, and 1 gal. of brine, contai 1 oz. of table salt, is added for each 5 lb. (original weight) of used. The mash is incubated for 30 to 90 days at a temperature

°C. (or 95 to 100°F.) and then strained. The residue is pressed, and extract is combined with the strainings. The liquor thus obtained is ted to just below the boiling point for 20 min. Alum (1 oz. to 80 gal. to 700 to 800 lb.) or kaolin (1 oz. to 1 gal.) is added to the heated uor. The mixture is permitted to settle overnight, after which it is red. The soy sauce obtained (12.5 lb. from 5 lb. of soybeans) is of first or highest grade. A second-grade sauce is obtained by adding water to the press cake (1.25 gal. to that from 5 lb. of soybeans), ing thoroughly, draining, and pressing the cake once more. Clarifica- of this liquor is achieved in the same manner as outlined for the -grade soy sauce. Then 0.75 lb. of table salt is added for each 5 lb. ginal weight) of soybeans used. A yield of 10 lb. of second-grade sauce may be obtained from each 5 lb. of soybeans. The first and nd grades of sauces may be mixed, and caramel may be added, if ed, to produce a darker color and to increase the viscosity.

*iemical Soy Sauce.* Soy sauce made by the chemical method is, for ractical purposes, a solution of amino acids and salts. It lacks the iols, nonnitrogenous organic acids, esters, aldehydes, and certain items which distinguish the fermented product. Chemical soy sauce be manufactured in the following manner (Lockwood and Smith, -1951): Soybean meal (or soybean-protein curd) is used as the raw rial. This is extracted, or washed, with water that has been adjusted acid to the isoelectric point of the soybean protein. Soluble mate- including carbohydrates and nitrogenous compounds, are removed. treatment increases the protein content of the residue, which is lyzed with 17 per cent HCl at 30 p.s.i. for 15 to 18 hr. The acid is ilized with sodium hydroxide or sodium carbonate. Should the acturer desire to obtain monosodium glutamate in addition to soy he incompletely neutralizes the acids and removes the crystals nosodium glutamate which form. Afterward neutralization is ted.

sauces made by the fermentation and chemical methods may be ned.

soy sauce made by the chemical method may be inoculated with l lactic acid cultures and yeasts and permitted to undergo a rela- short fermentation period.

*itions.* Various ingredients may be added to soy sauce, particu- he poorer grades, to improve their appearance and/or flavor and . Caramel may be added to enhance the color. Spices, such as all- cinnamon, clove, ginger, nutmeg, or pepper, may be added to e the flavor and aroma. Sugar may be added to increase the ss.

*iemical Changes.* During the fermentation, certain important take place. The enzymes of *A. oryzae* saccharify the starches, ze the proteins, and produce some alcohol. *L. delbrueckii* produces cid from some of the sugar. The yeast forms ethanol, carbon acetic acid, and other products from the sugar. Esters are from alcohols and acids. Aldehydes are produced. The amino acids, and their salts, including monosodium glutamate, contribu flavor of the sauce. Sodium chloride, present in a final concent 18 to 20 per cent in most samples, enhances the flavor and ai preservation of the product.

*Chemical Composition and Flavor.* Data on the chemical co of soy sauce have been presented by Wang and Ni (1936), Smit Lockwood and Smith (1950–1951), Onaga et al. (1957), and othe

Onaga and associates (1957) determined the chemical compo seven samples of soy sauce and evaluated them. The flavor wa to the amounts of salt, amino acids, nonnitrogenous acids, sug other flavoring agents present. Of the amino acids found, gluta alanine, leucine, and aspartic acid were present in larger amou arginine, glycine, lysine, methionine, phenylalanine, proline, serin nine, and valine. Alcohols, esters, and aldehydes were considered sible contributors to the aroma.

**Soy-sauce Yeasts.** Studies concerning the salt tolerance, su erance, and classification of osmophilic yeasts associated with so have been made by Onishi (1957).

**Tamari.** *Tamari* is a sauce prepared from soybeans, often w addition of other materials, such as rice. The flavor of *tamar* differs from that of soy sauce. This difference in flavor is due to of *A. tamarii*, this mold being the dominant microorganism in this The fermentation period is shorter than that of the soy-sauce ferme

**Miso.** *Miso* is the name given to products prepared from cook beans to which an *Aspergillus* starter and salt have been added and ing permitted. There are several different types of *miso*. The con tion of the salt used, the flavoring in the ingredients employed, a activity of the mold affect the type of final product. *Miso* is use cially as a breakfast food for children.

Smith (1949) has briefly described the oriental methods of usin beans as food, with special attention being given to the fer products.

**Koji.** *Koji* is the Japanese name given to starters, which ar in several fermentations, among which are the sake (rice wine), (soy sauce), *miso* (soy cheese), and *shocho* (distilled alcoholic fermentations. *Koji* may be named according to the purpose for it is used, for example, sake *koji* or *shoyu koji*. Its importance lies ability to digest starches and proteins with the production of products, which may subsequently be acted upon by yeasts or ba

The use of *koji* in the United States was introduced by Takamine

*Preparation of Koji.* *Koji* may be prepared in a number of wa from various substances. Generally a low-priced, bulky, fibrous ma such as the bran of rice or wheat, is used. The substance to be sterilized, cooled, and inoculated with the spores of a selected strain *A. flavus-oryzae* group. The inoculated material is incubated at a perature favorable to the growth of the mold until the enzymic a is at a maximum. Unless the *koji* is used immediately, the mold should then be stopped by drying.

n one method of making *koji*, described by Morikawa (1926), whole wn rice is used. The rice is cleaned, soaked for a few hours in water, then steamed. The grains are thus swelled and cracked or burst. The amed rice is spread out in trays in a layer approximately 0.5 in. thick ool. Spores of *A. oryzae* are mixed thoroughly with the rice, which then be heaped and covered with damp cloths to favor the growth of d mycelium. After incubation at 25 to 30°C. for a few hours, during ch the hyphae appear, the rice is again spread out in trays to a depth about 0.5 in. and incubated until the period of maximum enzyme luction.

uring incubation of the rice in the trays, it is necessary to regulate humidity carefully, for an excess of moisture may lead to bacterial lage of the molded rice, while insufficient moisture may not permit er development of the molds.

## Some References concerning Mold Enzymes

nces in *Enzymology*, Interscience Publishers, Inc., New York. (An annual publication.)
A. K., and E. F. Jansen: Proteolytic Enzymes, *Ann. Rev. Biochem.*, **21**: 1–28 1952). (Article contains 145 references.)
nn, E., und K. Myrbäck: "Die Methoden der Fermentforschung," Georg hieme Verlag, Stuttgart, 1941.
S. N., and D. R. Whitaker: Inhibition of Stimulation of the Cellulase of *Myroecium verrucaria*, *Arch. Biochem. and Biophys.*, **42**(1): 12–24 (1953).
. P. W.: Antibiotics Produced by Fungi, *Botan. Rev.*, **17**(6): 357–430 (1951).
ick, S. P., and N. O. Kaplan (eds.): "Methods in Enzymology," Vols. 1–3, reparation and Assay of Enzymes," Vol. 4, "Special Techniques," Academic ess, Inc., New York, 1955.
J. F., J. A. Johnson, and B. S. Miller: An Investigation of Commercial Fungal d Bacterial Alpha Amylase Preparations in Baking, *Cereal Chem.*, **27**: 191 950).
n. J., and A. F. Langlykke: Action of Mold Enzymes in Starch Saccharifican, *Cereal Chem.*, **25**: 190–201 (1948).
J., and G. L. Solomons: Enzymes from Micro-fungi, *Jour. Appl. Chem.*, 395–399 (1952).
*isse der Enzymforschung*, Akademische Verlagsgesellschaft m.b.H., Leipzig. n annual publication.)
, T. P. (ed.): Flexible Processing Keys Enzymes' Future, *Chem. Eng.*, **64**(2): –229 (1957).
H.: "Biochemie der Gärungen," Walter De Gruyter & Co., Berlin, 1952.
. T.: Preparation of *Aspergillus oryzae* Enzymes, *Ind. Eng. Chem.*, **23**: 1424 31).
C. G., H. W. Lincoln, and F. L. Gunderson: Purified Enzyme Preparations m *Aspergillus oryzae* in Bread Production, *Bakers Dig.*, **24**(6): 97–100 (1950).
. G. A.: Mold Enzymes Produced by Continuous Tray Method, *Food Inds.*, 688–690, 825, 826 (1948).
. J. A., and B. S. Miller: Fungal Enzymes Help You Make Better Bread, *d Eng.*, **23**: 161 (1951).
H. A. (ed.): "Respiratory Enzymes," Burgess Publishing Co., Minneapolis,
e, E. H., J. Corman, J. M. Van Lanen, and A. F. Langlykke: Production of d Amylase in Submerged Culture, *Jour. Bact.*, **54**: 149–159 (1947).
od, L. B.: *Trans. N.Y. Acad. Sci., Ser.* **2**, **15**: 1–5 (1952).
. G. R.: Localization of Carbohydrases at the Surface of Fungus Spores by Treatment, *Exptl. Cell Research*, **5**(1): 48–55 (1953).

McConnell, W. B., E. Y. Spencer, and J. A. Trew: The Proteolytic Microorganisms. V. Extracellular Peptidases Produced by Fungi Gr merged Culture, *Can. Jour. Chem.*, **31**: 697–704 (1953).

Mehler, A. H.: "Introduction to Zymology," Academic Press, Inc., 1957.

Morikawa, K.: A New Butyl and Isopropyl Alcohol Fermentation, P Massachusetts Institute of Technology, Cambridge, Mass., 1926.

Neilands, J. B., P. K. Stumpf, and R. Y. Stanier: "Outlines of Enzyme John Wiley & Sons, Inc., New York, 1955.

Onishi, H.: Studies on Osmophilic Yeasts. Part I. Salt-tolerance and Sug of Osmophilic Soy-yeasts, *Rept. Noda Inst. Sci. Research Japan,* pp. 16–21.

———: Part II. Factors Affecting Growth of Soy Yeasts and Others in th ment of a High Concentration of Sodium Chloride, *Rept. Noda In search Japan,* No. 1, 1957, pp. 22–29.

———: Part III. Classification of Osmophilic Soy and Miso Yeasts, *R Inst. Sci. Research Japan,* No. 1, 1957, pp. 30–35.

Pool, E. L., and L. A. Underkofler: Fungal Saccharifying Agents Amyloly of Bran Culture and Submerged Culture, *Jour. Agr. Food Chem.,* 1 (1953).

Prescott, S. C., and C. G. Dunn: "Industrial Microbiology," 2d ed., Mc Book Company, Inc., New York, 1949, pp. 673–675.

Reed, G.: Enzyme Technology: A Process Frontier, *Chem. Inds. Week* 1950.

———: Industrial Enzymes . . . Now Speed Natural Processes, *Food En* 105–109 (1952*a*).

———: Fungal Enzymes in Bread Baking, *Food Technol.,* **6**(9): 339–341 (

Rivett, R. W., M. J. Johnson, and W. H. Peterson: Laboratory Ferm Aerobic Fermentations, *Ind. Eng. Chem.,* **42**: 188–190 (1950).

Roy, D. K., and L. A. Underkofler: Purification of Fungal Maltase, *Cere* **28**(1): 72–76 (1951).

Shu, P., and A. C. Blackwood: Studies on Carbon and Nitrogen Source Production of Amylolytic Enzymes by Submerged Culture of *Aspergi* *Can. Jour. Botany,* **29**: 113–124 (April, 1951).

Simpson, F. J.: Microbial Pentosanases. I. A Survey of Microorganism Production of Enzymes That Attack the Pentosans of Wheat Flour, *C Microbiol.,* **1**: 131–139 (1954).

Smythe, C. V.: Microbiological Production of Enzymes and Their Industr cations, *Econ. Botany,* **5**(2): 126–144 (1951). (This article contains ences.)

Snyder, E. G.: New Enzymes "Open New Doors," *Food Eng.,* **25**(6): 8 (1953).

Stetter, H.: "Enzymatische Analyse," Verlag Chemie, G.m.b.H., Weinheim Darmstadt, Germany, 1951.

Sumner, J. B., and K. Myrbäck: "The Enzymes, Chemistry and Mech Action," Academic Press, Inc., New York, 1951.

——— and G. F. Somers: "Chemistry and Methods of Enzymes," Academ Inc., New York, 1953.

Takamine, J.: Enzymes of *Aspergillus oryzae* and the Application of Its Am Enzyme to the Fermentation Industry, *Ind. Eng. Chem.,* **6**: 824 (1914).

Tauber, H.: "The Chemistry and Technology of Enzymes," John Wiley & S New York, 1949.

Toyama, N.: A Crystalline Enzyme from the Mold *Trichoderma konin* *Miyazaki, Bull. Fac. Agr.,* **2**(1 and 2): 79–81 (1957).

Tsuchiya, H. M., J. Corman, and H. J. Koepsell: II. Factors Affecting the tion of Alpha-amylase and Maltase by Certain Aspergilli, *Cereal Chem* 322–330 (1950).

Underkofler, L. A., and W. J. Ferracone: Commercial Enzymes—Potent C that Promote Quality, *Food Eng.,* **29**(4): 123 (1957).

— and R. J. Hickey: "Industrial Fermentations," Chemical Publishing Company, Inc., New York, 1954.

— and D. K. Roy: Crystallization of Fungal Alpha-amylase and Limit Dextrinase, *Cereal Chem.*, **28**(1): 18–29 (1951).

Production and Marketing Administration, *U.S. Dept. Agr., Tech. Bull.* 1024, 1950.

Lanen, J. M., and E. H. Le Mense: The Production of Fungal Amylases in Submerged Culture and Their Use in the Production of Industrial Alcohol, *Jour. Bact.*, **51**: 595 (1946).

tson, R. W.: Research on Industrial Enzymes, *Can. Chem. Processing*, December, 1953.

ter, L. R.: The Proteolytic Enzymes of Microorganisms. IV. Partial Purification and Some Properties of Extracellular Protease from *Mortierella renispora* Dixon-Stewart, *Can. Jour. Botany*, **30**: 685–692 (1952).

—: VI. The Separation of Proteases from *Mortierella renispora* Dixon-Stewart by Zone Electrophoresis, *Can. Jour. Biochem. and Biophys.*, **32**: 20–26 (1954*a*).

—: VII. A Study of the Properties of Two Proteases Isolated from *Mortierella renispora* Dixon-Stewart, *Can. Jour. Biochem. and Biophys.*, **32**: 60–67 (1954*b*).

taker, D. R.: Purification of the Cellulase of *Myrothecium verrucaria, Nature*, **168**: 1070 (1951).

—: An Effect of Proteins and Proteoses on the Cellulase of *Myrothecium verrucaria, Science*, **116**(3004): 90–92 (1952).

—: Mutarotation after Hydrolysis of Cellopentaose by *Myrothecium verrucaria* Cellulase, *Arch. Biochem. and Biophys.*, **53**(2): 436–438 (1954*a*).

—: Hydrolysis of a Series of β-1,4-Oligoglucosides by *Myrothecium verrucaria* Cellulase, *Arch. Biochem. and Biophys.*, **53**(2): 439–449 (1954*b*).

—, J. R. Colvin, and W. H. Cook: The Molecular Weight and Shape of *Myrothecium verrucaria* Cellulase, *Arch. Biochem. and Biophys.*, **49**(2): 257–262 (1954).

## ome References concerning Soy Sauce and Fermented Soybean Foods

h. M. B.: Soy and Related Fermentations, *U.S. Dept. Agr., Bull.* 1152, 1923.

ood, L. B.: The Production of Chinese Soy Sauce, *Soybean Dig.*, **7**(12): 10, 11 947).

and A. K. Smith: Fermented Soy Foods and Sauce, "Yearbook of Agriculture, 50–51," U.S. Department of Agriculture, pp. 357–361.

D. M., B. S. Luh, and S. J. Leonard: Quality Evaluation and Chemical Com-sition of Soy Sauce, *Food Research*, **22**(1): 83–88 (1957). (This article contains references, several of which relate to the Japanese literature and which are ncerned with flavor and chemical composition.)

A. K.: Oriental Methods of Using Soybeans as Food with Special Attention Fermented Products, *U.S. Dept. Agr., Bur. Agr. and Ind. Chem., Mimeo-phed Circ. Ser., AIC* 234, June, 1949.

T. H., and S. M. Ni: Chemical Studies on the Manufacture of Soybean Sauce the Kwanton Process, *Jour. Chem. Eng. Natl. Chekiang Univ.*, **3**: 284–296 936).

## Some Patents

, R. R.: Removal of Sugars by Enzymatic Process, U.S. Patent 2,744,017. v 1, 1956.

V. G.: Process of Manufacturing Vegetable Glue, U.S. Patents 1,257,307, Feb. 1918; 1,273,571, July 23, 1918.

A., and J. Effront: Process for Treating Amylaceous Substances, U.S. Patent 7,374, May 22, 1917.

nd ———: Process of Manufacturing Diastase and Toxins by Oxidizing Fer-ts, U.S. Patent 1,227,525, May 22, 1917.

——— and ———: Method of Producing Bacterial Enzyme Prepara
Patent 1,744,742, Jan. 28, 1930.

Douglas, R.: Process of Preparing Pectin, U.S. Patent 1,858,820, May 17,

Fränkel, S.: Manufacture of Diastase, U.S. Patent 1,129,387, Feb. 23, 1915.

Luther, H. G.: Treatment of Flour with Glucose Oxidase, U.S. Patent
Feb. 26, 1957.

Paine, H. S., and J. Hamilton: Process for Preparing Fondant or Choc
Cream Centers, U.S. Patent 1,437,816, Dec. 5, 1922.

Takamine, J.: Preparing and Making Fermented Alcoholic Liquors, U
525,819, Sept. 11, 1894.

———: Preparing and Making Taka-Koji, U.S. Patent 525,820, Sept. 11, 189

———: Preparing and Making Moto, U.S. Patent 525,821, Sept. 11, 1894.

———: Preparing and Making Taka-Moyashi, U.S. Patent 525,822, Sept. 1

———: Process of Making Diastatic Enzyme, U.S. Patent 525,823, Sept. 11

———: Taka-Koji Ferment and Process of Making the Same, U.S. Paten
Sept. 11, 1894.

———: Means for and Method of Converting Starch Material into Su
Patent 525,971, Sept. 11, 1894.

———: Process of Converting Starchy Material into Sugar, U.S. Patent
June 16, 1896.

———: Enzym, U.S. Patent 991,560, May 9, 1911.

———: Amylolytic Enzym, U.S. Patent 991,561, May 9, 1911.

———: Process for Producing Diastatic Product, U.S. Patent 1,054,324,
1913.

———: Diastatic Product, U.S. Patent 1,054,626, Feb. 25, 1913.

———: Diastatic Product and the Process for Producing the Same, U.S
1,148,938, Aug. 3, 1915.

———: Process for Producing Diastatic Product, U.S. Patent 1,263,817, Apr.

——— and J. Takamine, Jr.: Enzymic Substance and Process of Making th
U.S. Patents 1,391,219, Sept. 20, 1921; 1,460,736, July 3, 1923.

Wallerstein, L.: Beer and Method of Preparing Same, U.S. Patent 995,820,
1911.

———: Preparation for Use in Brewing, U.S. Patent 995,823, June 20, 1911.

———: Method of Treating Beer or Ale, U.S. Patents 995,824; 995,826, June

———: Method of Treating Beer or Ale, U.S. Patent 997,873, July 11, 1911.

———: Invertase Preparation and Method of Making the Same, U.S.
1,919,675; 1,919,676, July 25, 1933.

———: Process of Maturing and Ripening Beers and Ales, U.S. Patent 2
Apr. 20, 1937.

———: Process of Chillproofing and Stabilizing Beers and Ales, U.S. Patent
447; 2,077,448, Apr. 20, 1937.

———: Process of Chillproofing and Stabilizing Malt Beverages, U.S. Paten
449, Apr. 20, 1937.

———: Rubber, U.S. Patent 2,097,481, Nov. 2, 1937.

———: Deproteinization of Rubber Latex, U.S. Patent 2,116,089, May 3, 193

Wallerstein, M.: Process of Making Chocolate Sirup, U.S. Patents 1.
1,854,355, Apr. 19, 1932.

## CHAPTER 42

# SOME MINOR CHEMICAL ACTIVITIES OF THE LOWER FUNGI

e of the most interesting aspects of microbiology is the biochemistry
olds. Because of their ubiquity and the mischief these organisms,
regarded as biological outcasts, can do in many branches of indus-
their study has distinct economic importance. This study of the
r fungi has advanced rapidly since about 1922. The outstanding
of Raistrick and his associates is mentioned in particular, because
e new and careful methods of investigation they have devised. A
number of papers, many of which are cited at the end of this
ter, have been published on various aspects of this organized re-
h.

tudy of Table 145 (page 524) will give the reader some idea of the
sified metabolic products formed by the lower fungi. Methods
roduction of some of the most important substances mentioned
s table have been discussed in some detail in the preceding chap-
The scope of this text does not permit extended consideration of
.

he pages which follow is summarized information relating to prod-
ormed by some molds. Table 195 lists some closely related acids,
e compounds, and pigments. Table 196 gives the names of some
and their structural formulas, together with the names of some of
lds capable of producing them.

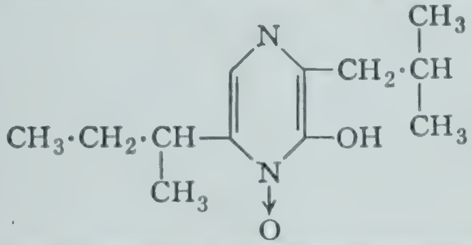
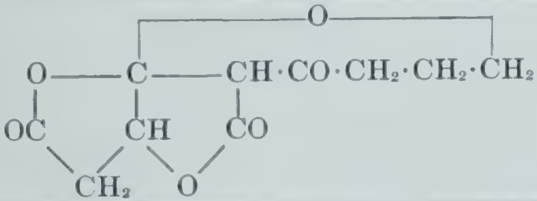
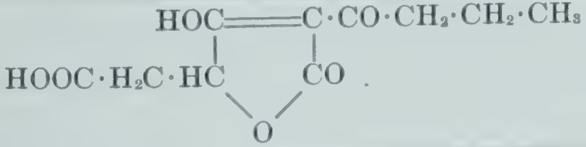
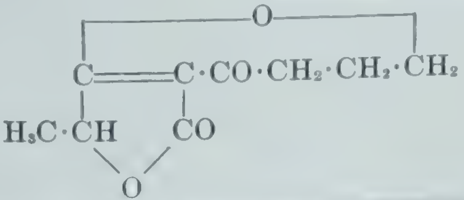
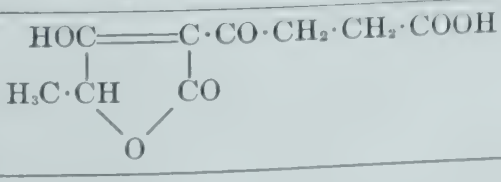
n the metabolic products of *Penicillium charlesii* G. Smith several
c acids have been isolated. Carolic, carolinic, carlic, and carlosic
were first described by Clutterbuck and his associates (1934). The
wo of these acids are dextrorotatory, while the last two are levo-
ry. They may be obtained by growing the mold on the Czapek-Dox
m, but larger yields of carolinic acid are obtained on the Raulin-
medium, while no carlosic acid has been isolated from the latter
n. Carolic and carolinic acids (1935) are derivatives of *l*-gamma-
tetronic acid and are structurally closely related to ascorbic acid.
and carlosic acids are derivatives of *l*-gamma-carboxymethyltet-
cid, also of *l*-γ-methyltetronic acid, and are structurally closely
to carolic and carolinic acids and also to ascorbic acid. *l*-γ-Methyl-
acid is also a product of *P. charlesii* G. Smith.

lisporic and minioluteic acids are complex fatty acids.

TABLE 195. SOME GROUPS OF CLOSELY RELATED COMPOUNDS PRODUCED B

| Acids | Benzene compounds | Pigmen |
|---|---|---|
| **Citric acid derivatives** | **Compounds with 1 carbon chain** | **Anthraquinone** |
| Agaricic acid | Anisaldehyde | Catenarin |
| Itaconic acid | Gentisic acid | Chrysophanol |
| Gamma-ketopentadecoic acid | Gentisyl alcohol | Cynodontin |
| Minioluteic acid | Methylanisate | Emodic acid |
| Spiculisporic acid | Methylcinnamate | Erythroglaucin |
| **Tetronic acid derivatives** | Methyl *p*-methoxycinnamate | *Frangula*-emodin |
| Ascorbic acid | **Compounds with 2 carbon chains** | Helminthosporin |
| Carlic acid | 3,5-Dihydroxyphthalic acid | Omega-hydroxye |
| Carlosic acid | 6-Hydroxy-2-methylbenzoic acid | Islandicin |
| Carolic acid | Ustic acid | Nalgiolaxin |
| Carolinic acid | | Nalgiovensin |
| Dehydrocarolic acid | | Physcion |
| *l*-γ-Methyltetronic acid | | Roseopurpurin |
| Penicillic acid | | Tritisporin |
| Terrestric acid | | **Naphthaquin derivative** |
| **Tropolone derivatives** | | Javanicin |
| Puberulic acid | | Oxyjavanicin |
| Puberulonic acid | | **Toluquinon derivatives** |
| Stipitatic acid | | Fumigatin |
| | | 4-Methoxy-2,5-tolu none |
| | | Oosporein |
| | | Phoenicin |
| | | Spinulosin |
| | | **Xanthone deriv** |
| | | Ravenelin |
| | | Rubrofusarin |

TABLE 196. SOME ACIDS FORMED BY MOLDS

| Acid | Formula | Produced by |
|---|---|---|
| itic(69) *<br>$H_6O_6$) | $\begin{array}{l} CH_2\cdot COOH \\ \vert \\ C\cdot COOH \\ \Vert \\ CH\cdot COOH \end{array}$ | *Aspergillus itaconicus* |
| ic<br>$_{40}O_7$) | $\begin{array}{l} CH_3 \\ \vert \\ (CH_2)_{15} \\ \vert \\ CH\cdot COOH \\ \vert \\ C(OH)\cdot COOH \\ \vert \\ CH_2 \\ \vert \\ COOH \end{array}$ | *Polyporus officinalis*<br>*Fomes laricis* |
| illic<br>$O_2N_2$) |  | *A. flavus* |
| ydrous)(36, 43)<br>$_{10}O_6$) |  | *Penicillium charlesii* G. Smith |
| (36, 43)<br>$_{12}O_6$) |  | *P. charlesii* G. Smith |
| drous)(36, 41)<br>$O_4$) |  | *P. charlesii* G. Smith |
| c(36, 41)<br>$O_6\cdot H_2O$) |  | *P. charlesii* G. Smith |
| $)_7$) | $\begin{array}{l} CH_2\cdot COOH \\ \vert \\ C(OH)\cdot COOH \\ \vert \\ CH_2\cdot COOH \end{array}$ | *A. niger* |

---

numbers in parentheses refer to the citations at the end of the chapter.

TABLE 196. SOME ACIDS FORMED BY MOLDS—(*Continued*)

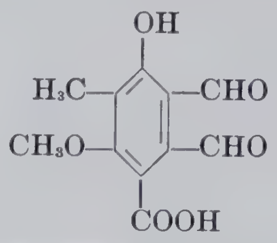
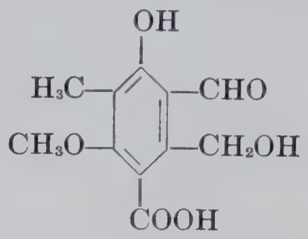
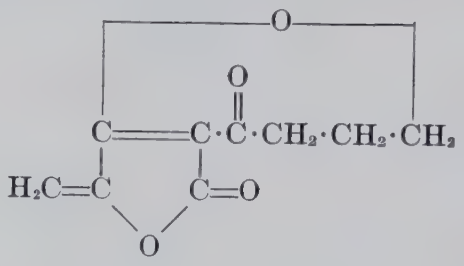
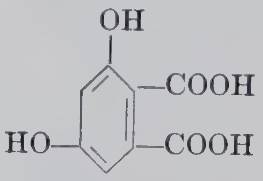
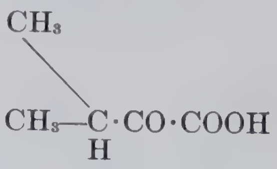
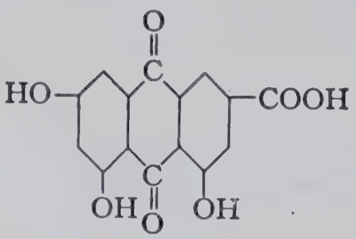
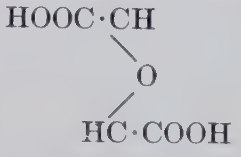
| Acid | Formula | Prod |
|---|---|---|
| Cyclopaldic(85) |  | *P. cyclo*<br>*album* |
| Cyclopolic(85) |  | *P. cyclop*<br>*album* |
| Dehydrocarolic ($C_9H_8O_4$)(75) |  | *P. cineras*<br>Biourge |
| 3,5-Dihydroxyphthalic(25) ($C_8H_6O_6$) |  | Some spec<br>strains of<br>*brevi-com*<br>series |
| Dimethyl pyruvic ($C_5H_8O_3$) |  $CH_3-\overset{CH_3}{\underset{H}{C}}\cdot CO\cdot COOH$ | *A. niger* |
| Emodic(64) |  | *P. cyclopiu*<br>Westling |
| *l*-Ethylene oxide α, β dicarboxylic ($C_4H_4O_5$) |  | *Monilia for*<br>*P. viniferum* |
| Formic ($CH_2O_2$) | $H\cdot COOH$ | *A. oryzae* |

TABLE 196. SOME ACIDS FORMED BY MOLDS—(*Continued*)

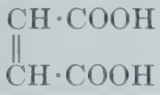
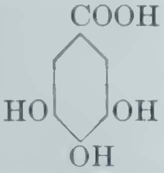
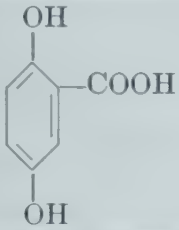
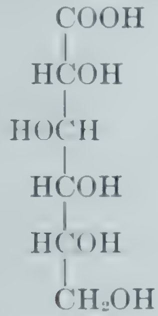
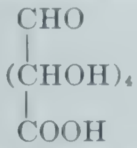
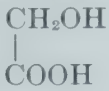
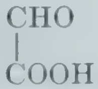
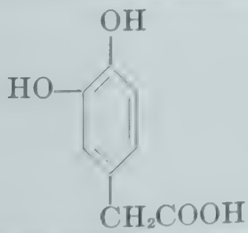
| Acid | Formula | Produced by |
|---|---|---|
| .[44]<br>,$H_{12}O_8$) | | *P. griseo-fulvum* Dierckx<br>*P. flexuosum* Dale<br>*P. brefeldianum* Dodge |
| ric<br>$H_4O_4$) |  $\begin{array}{l} CH \cdot COOH \\ \Vert \\ CH \cdot COOH \end{array}$ | Species of the genera *Rhizopus, Mucor,* etc. |
| (3,4,5-tri-<br>roxybenzoic)<br>$_6O_5$) |  | *A. niger*<br>See Chap. 35 |
| ic[29] (2:5-di-<br>roxybenzoic)<br>$_6O_4$) |  | *P. griseo-fulvum* Dierckx |
| nic<br>$_{12}O_7$) |  $\begin{array}{c} COOH \\ \vert \\ HCOH \\ \vert \\ HOCH \\ \vert \\ HCOH \\ \vert \\ HCOH \\ \vert \\ CH_2OH \end{array}$ | *A. niger*<br>*P. chrysogenum,* etc.<br>See Chap. 29 |
| nic<br>$O_7$) |  $\begin{array}{c} CHO \\ \vert \\ (CHOH)_4 \\ \vert \\ COOH \end{array}$ | *Ustulina vulgaris* |
| $O_3$) |  $\begin{array}{c} CH_2OH \\ \vert \\ COOH \end{array}$ | *A. niger* |
| ic<br>$)_3$) |  $\begin{array}{c} CHO \\ \vert \\ COOH \end{array}$ | *A. niger* |
| nto-<br>nic<br>$)_4$) |  | *Polyporus tumulosus* |

TABLE 196. SOME ACIDS FORMED BY MOLDS—(*Continued*)

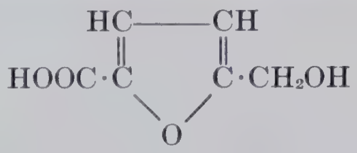
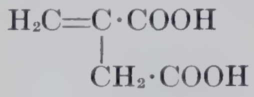
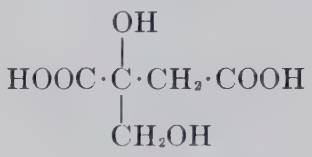
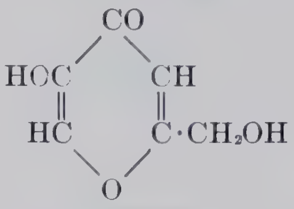
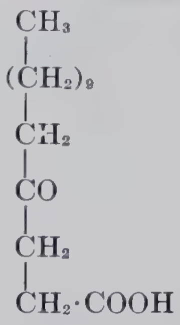
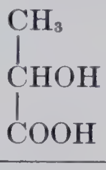
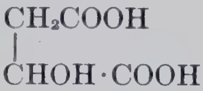
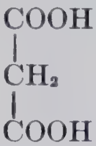
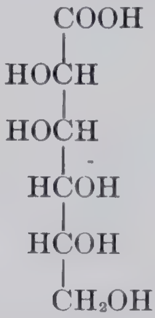
| Acid | Formula | Prod |
|---|---|---|
| 2-Hydroxymethylfurane-5-carboxylic ($C_6H_6O_4$) |  | *A. glauc*<br>*A. clavat*<br>*A. niger*<br>*A. oryzae*<br>*A. wentii* |
| Itaconic⁽⁶³⁾ ($C_5H_6O_4$) |  $H_2C{=}C\cdot COOH$, $\vert$ $CH_2\cdot COOH$ | *A. itacon*<br>*A. terreus*<br>See Chap |
| Itatartaric ($C_5H_8O_6$) |  $HOOC\cdot C(OH)(CH_2OH)\cdot CH_2\cdot COOH$ | *A. terreus* |
| Kojic ($C_6H_6O_4$) |  | *A. flavus*,<br>See Chap |
| γ-Ketopentadecoic ($C_{15}H_{28}O_3$) |  $CH_3\text{—}(CH_2)_9\text{—}CH_2\text{—}CO\text{—}CH_2\text{—}CH_2\cdot COOH$ | *P. spiculis*<br>Lehman |
| Lactic ($C_3H_6O_3$) |  $CH_3\text{—}CHOH\text{—}COOH$ | Species of<br>era *Rhiz*<br>and *Muc*<br>See Chap. |
| Malic ($C_4H_6O_5$) |  $CH_2COOH\text{—}CHOH\cdot COOH$ | *A. fumaricu*<br>*A. niger*, et |
| Malonic ($C_3H_4O_4$) |  $COOH\text{—}CH_2\text{—}COOH$ | *P. funiculos* |
| D-Mannonic ($C_6H_{12}O_7$) |  $COOH\text{—}HOCH\text{—}HOCH\text{—}HCOH\text{—}HCOH\text{—}CH_2OH$ | *P. purpuro*<br>var. *rubr*<br>*rotium* |

TABLE 196. SOME ACIDS FORMED BY MOLDS—(*Continued*)

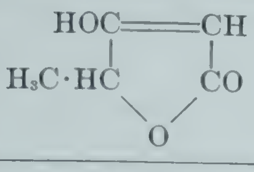
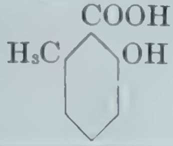
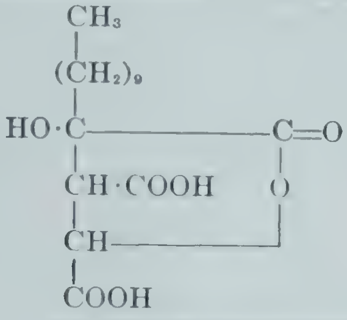
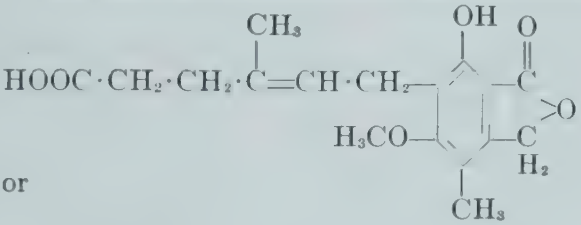
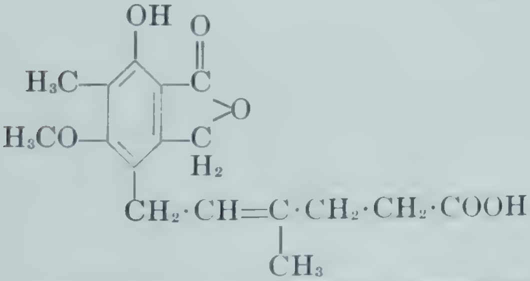
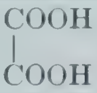
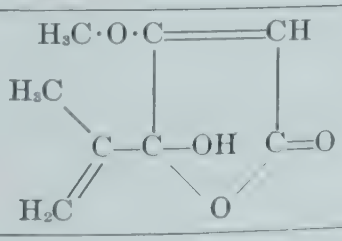
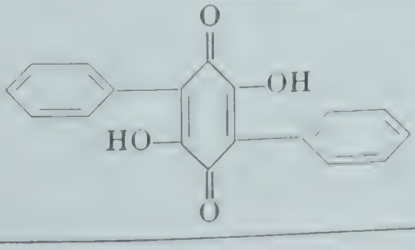
| Acid | Formula | Produced by |
|---|---|---|
| ethyl-<br>onic(45)<br>$H_6O_3$) |  | *P. charlesii* G. Smith |
| thylsali-<br>c(19)<br>ydroxy-6-<br>hylbenzoic)<br>$H_8O_3$) |  | *P. griseo-fulvum* Dierckx<br>*P. flexuosum* Dale |
| luteic(38)<br>$H_{20}O_7$) |  | *P. minio-luteum* Dierckx |
| henolic(31, 86)<br>$H_{20}O_6$) | <br>or<br> | Some species and strains of the *P. brevi-compactum* series |
| $O_4$) |  $\mathrm{COOH}-\mathrm{COOH}$ | *A. niger*, etc. |
| (45)<br>$O_4$) |  | *P. puberulum* Bainier<br>*P. cyclopium* Westling |
| 3 |  | *Polyporus nidulans* |

Table 196. Some Acids Formed by Molds—(*Continued*)

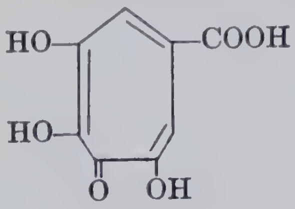
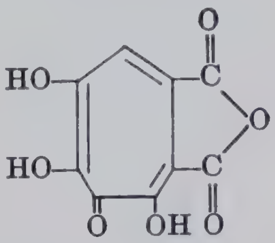
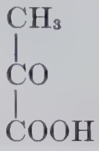
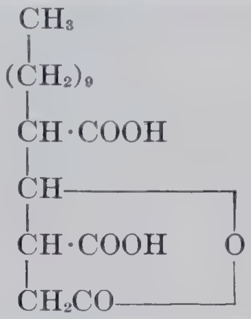
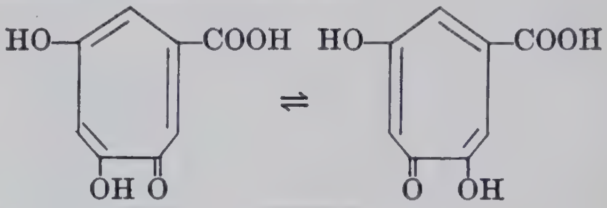
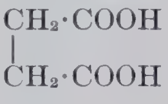
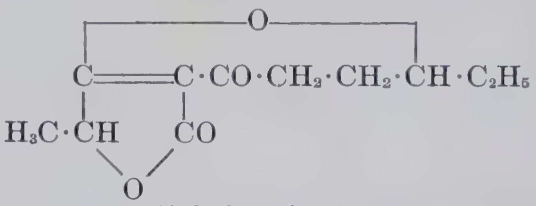
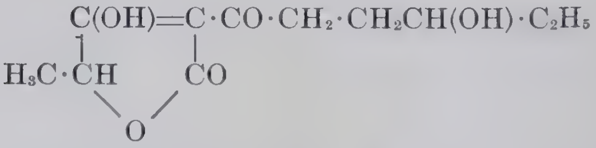
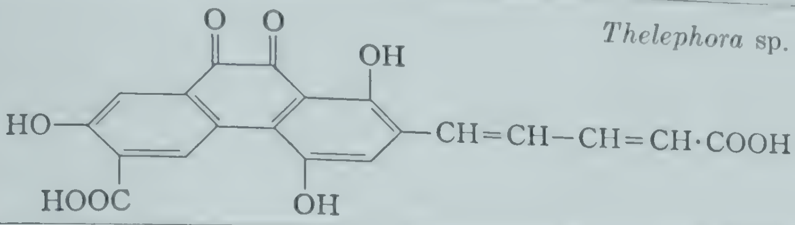
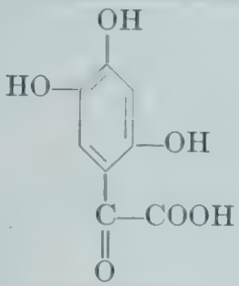
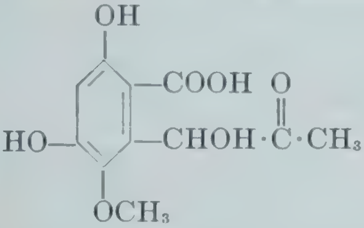
| Acid | Formula | Pro |
|---|---|---|
| Puberulic(23)<br>($C_8H_6O_6$) |  | *P. aura*<br>Biour<br>*P. pube*<br>Baini<br>*P. Joha*<br>Zalesk |
| Puberulonic<br>($C_9H_4O_7$) |  | *P. pube*<br>Bainie |
| Pyruvic (pyro-racemic)<br>($C_3H_4O_3$) |  $CH_3{-}CO{-}COOH$ | *A. niger* |
| Spiculisporic(16, 38)<br>($C_{17}H_{28}O_6$) |  | *P. spiculi*<br>Lehma<br>*P. crateri*<br>Gilman<br>Abbott<br>*P. minio-l*<br>Dierckx |
| Stipitatic(70)<br>($C_8H_6O_5$) |  | *P. stipitat*<br>Thom |
| Succinic<br>($C_4H_6O_4$) |  $CH_2{\cdot}COOH{-}CH_2{\cdot}COOH$ | Species of<br>era *Rhi*<br>*Mucor,*<br>*rium,* et |
| Terrestric(52)<br>($C_{11}H_{14}O_4$) | <br>(Anhydrous form)<br><br>(Hydrate form) | Strains of<br>*restre*<br>Jensen ser |

TABLE 196. SOME ACIDS FORMED BY MOLDS—(*Continued*)

| Acid | Formula | Produced by |
|---|---|---|
| phoric<br>$H_{12}O_9$) |  | *Thelephora* sp. |
| Trihydroxy-<br>nylglyoxylic<br>$H_6O_6$) |  | *Polyporus tumulosus* |
| 2)<br>$H_{12}O_7$) |  | *A. ustus* |

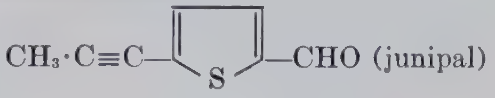
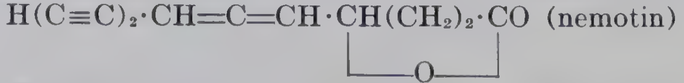
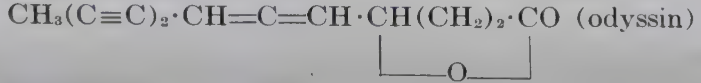
## ACETYLENIC COMPOUNDS

ock (1956) has reviewed the occurrence of acetylenic compounds ıral products, while he and his associates have studied the producd chemistry of a number of these compounds from the higher fungi. rding to Bu'Lock, acetylenic compounds are formed by certain plants, basidiomycetes, and at least one actinomycete. Acetylenic unds containing 10, 12, 13, 14, and 17 carbon atoms have been isorom the essential oils of some groups of Compositae and Umbel- those containing 18 carbon atoms, from the seed oil of some erms; and those containing 8, 9, 10, 11, 12, and 13 carbon atoms, ıngi.

i, which are known to produce acetylenic compounds, belong to s Basidiomycetes, the order Agaricales, and the family Agaricahich includes species of the genera *Agrocybe*, *Clitocybe*, *Coprinus*, *ila*, and *Marasmius*) and the family Polyporaceae (which inpecies of the genera *Daedalea*, *Fistulina*, *Polyporus*, and *Poria*). ples of some of the types of acetylenic compounds formed by the ungi and an actinomycete species are shown in Table 197.

ck, Jones, and Turner (1957) found that *Polyporus anthra-* formed a complex and variable mixture of at least 17 acetylenic ites. These compounds were ethylenic-acetylenic alcohols, acids,

TABLE 197. EXAMPLES OF SOME ACETYLENIC COMPOUNDS FORMED BY MICROBES

| Producing microorganism | Structural formula | No. of carbon atoms | Reference |
|---|---|---|---|
| *Agrocybe dura* | $HO\cdot CH_2\cdot (C\equiv C)_3\cdot CO\cdot NH_2$ (agrocybin) | 8 | Kavanagh et al.: *Proc. Nat. Acad. Sci.*, **36**: 102 (1950). |
| *Clitocybe diatetra* | $HOOC\cdot CH{=}CH\cdot (C\equiv C)_2\cdot CO\cdot NH_2$<br>$HOOC\cdot CH{=}CH\cdot (C\equiv C)_2\cdot CN$ | 8<br>8 | Anchel: *Jour. Am. Chem. Soc.*, **74**: 1588 (1952); **75**: 4621 (1953); *Science*, **121**: 607 (1955). |
| *Daedalea juniperina* |  (junipal) | 8 | Birkinshaw and Chaplen: *Biochem. Jour.*, **60**: 255 (1955). |
| *Polyporus anthracophilus* | $HO_2C\cdot CH{=}CH\cdot C\equiv C\cdot CH{=}CH\cdot CO_2H$<br>$CH_3\cdot CH{=}CH\cdot (C\equiv C)_2\cdot CH{=}CH\cdot CH_2OH$<br>$HO\cdot (CH_2)_3\cdot (C\equiv C)_2\cdot CH{=}CH\cdot COOR$ * | 8<br>10<br>10 | Bu'Lock et al.: *Chemistry & Industry*, 1955, p. 686.<br>———: *Quart. Revs. London*, **10**: 371 (1956). |
| *P. fumosus* * | $RO_2C\cdot (CH_2)_2\cdot (C\equiv C)_2\cdot CH{=}CH\cdot CO_2R$ †<br>$CH_3\cdot CH{=}CH\cdot (C\equiv C)_2\cdot CH{=}CH\cdot CO_2R$ †<br>$RO_2C\cdot CH{=}CH\cdot (C\equiv C)_2\cdot CH{=}CH\cdot CO_2R$ † | 10<br>10<br>10 | |
| *Basidiomycete* B.841 | $H\cdot (C\equiv C)_2\cdot CH{=}C{=}CH\cdot CH(OH)\cdot (CH_2)_2COOH$ (nemotinic acid)<br> $H(C\equiv C)_2\cdot CH{=}C{=}CH\cdot \underbrace{CH(CH_2)_2\cdot CO}_{O}$ (nemotin)<br>$CH_3(C\equiv C)_2\cdot CH{=}C{=}CH\cdot CH(OH)\cdot (CH_2)_2COOH$ (odyssic acid)<br> $CH_3(C\equiv C)_2\cdot CH{=}C{=}CH\cdot \underbrace{CH(CH_2)_2\cdot CO}_{O}$ (odyssin) | 11<br>11<br>12<br>12 | Bu'Lock et al.: *Jour. Chem. Soc.*, 1955, p. 4270.<br>——— and Leadbeater: *Biochem. Jour.*, **62**: 476 (1956).<br>——— et al.: *Jour. Chem. Soc.*, 1957, p. 1097. |

roxy-acids, and diacids. The acids occurred free, as methyl esters, as esters with the hydroxy-compounds of the series.

## PIGMENTS

veral different types of coloring matter have been isolated from ls. Hydroxyanthraquinones are produced by species of *Helminthoium*. For example, a mixture of helminthosporin and catenarin conted about 30 per cent of the dried weight of the mycelium of *H. ineum* Rabenhorst, there being 2 to 3 parts of the former substance part of the latter. Cynodontin and tritisporin are other hydroxyraquinones produced by species of *Helminthosporium*. Certain dyes may be manufactured from alpha-hydroxyanthraquinones.

ta concerning some pigments of the anthraquinone group, which are uced by molds, are presented in Table 198.

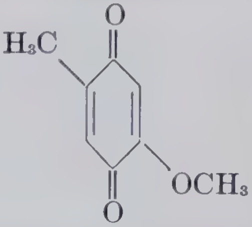
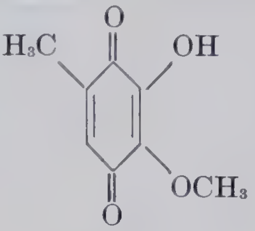
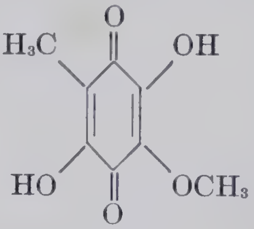
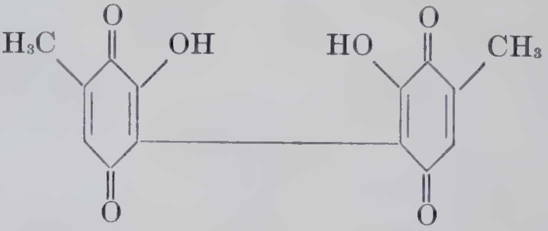
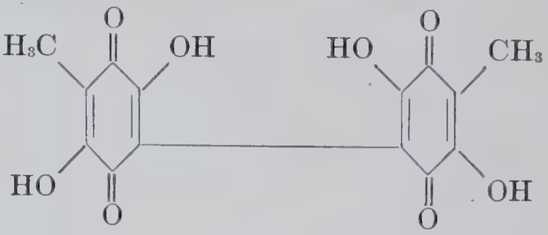
e structural formulas of some mold pigments which are derivatives luquinone are given in Table 199.

BLE 198. SOME PIGMENTS OF THE ANTHRAQUINONE GROUP PRODUCED BY MOLDS

| Pigment | Formula | Color | Mold producing |
|---|---|---|---|
| arin ........... | $C_{15}H_{10}O_6$ | Red | *Helminthosporium catenarium*<br>*H. gramineum*<br>*H. velutinum*<br>*H. tritici-vulgaris* |
| phanol ........ | $C_{15}H_{10}O_4$ |  | *Penicillium islandicum* |
| ontin .......... | $C_{15}H_{10}O_6$ | Bronze | *H. cynodontis*<br>*H. euclaenae*<br>*H. avenae* |
| acid ......... | $C_{15}H_8O_7 \cdot H_2O$ | Orange | *P. cyclopium* |
| glaucin ....... | $C_{16}H_{12}O_6$ | Dark red | *Aspergillus glaucus* |
| *la*-emodin ..... | $C_{15}H_{10}O_5$ | Red (blood) | *Dermocybe sanguinea* Wulf |
| thosporin ..... | $C_{15}H_{10}O_5$ | Dark maroon | *H. catenarin*<br>*H. cynodontis*<br>*H. gramineum*<br>*H. tritici-vulgaris* |
| roxyemodin .... | $C_{15}H_{10}O_6$ | Orange | *P. cyclopium*<br>*P. citreo-roseum* |
| in ............ | $C_{15}H_{10}O_5$ | Dark red | *P. islandicum* |
| axin * ........ | $C_{18}H_{15}O_6Cl$ | Yellow | *P. nalgeovensis* |
| ensin * ....... | $C_{18}H_{16}O_6$ | Orange | *P. nalgeovensis* |
| n ............. | $C_{16}H_{12}O_5$ |  | *A. glaucus* series |
| rpurin ....... | $C_{16}H_{12}O_6$ | Red | *P. roseo-purpureum* |
| rin * ......... | $C_{15}H_{12}O_7$ | Red | *H. tritici-vulgaris* |

ture not fully elucidated.

Table 199. Some Mold Pigments Which Are Toluquinone De

| Pigment | Structural formula | P |
|---|---|---|
| 4-Methoxy-2,5-toluquinone<br>$(C_8H_8O_3)$<br>(orange yellow) |  | *Copr*<br>*Lenti* |
| Fumigatin<br>(3-Hydroxy-4-methoxy-2,5-toluquinone)<br>$(C_8H_8O_4)$<br>(maroon red) |  | *Asper*<br>*gati* |
| Spinulosin<br>(3,6-Dihydroxy-4-methoxy-2,5-toluquinone)<br>$(C_8H_8O_5)$<br>(purple black) |  | *Penicil*<br>*losur*<br>*P. cine*<br>*A. fum* |
| Phoenicin<br>$(C_{14}H_{10}O_6)$<br>(red) |  | *P. phoe*<br>*P. rubr* |
| Oösporein<br>$(C_{14}H_{10}O_8)$ |  | *Oöspor*<br>*(Geo*<br>*color* |

## MOLD POLYSACCHARIDES

Some data relating to the mold polysaccharides produced fro are tabulated in Table 200.

## SOME MISCELLANEOUS PRODUCTS

**Acetaldehyde.** By use of fixation methods, acetaldehyde recovered from media fermented by species of the genera As *Monilia, Mucor, Geotrichum, Penicillium,* and other molds.

TABLE 200. SOME MOLD POLYSACCHARIDES PRODUCED FROM GLUCOSE *

| Polysaccharide | Products of hydrolysis | Produced by |
|---|---|---|
| eolinose | Galactose, glucose, malonic acid, and mannose | *Penicillium capreolinum* |
| tocarolose | D-Galactose | *P. charlesii* G. Smith |
| gen | Glucose | A white species of *Aspergillus* |
| acid | Glucose and malonic acid | *P. luteum* Zukal |
| ocarolose | D-Mannose | *P. charlesii* G. Smith |
| starch | Glucose | *A. fumigatus, A. glaucus, A. niger, A. oryzae, P. glaucum, P. variabile* |
| lextrin | Glucose | *A. niger, P. expansum* |
| ose | Galactose | *P. rugulosum* |
| tiose | Glucose | *P. sclerotiorum* |
| ose | D-Galactose, D-Glucose, and either D-iodose or L-altrose | *P. variens* G. Smith |

W. Cluttterbuck, *Jour. Soc. Chem. Ind.*, **55**: 55T (1936).

**yl Acetate.** This substance is produced by *P. digitatum* Sacc. from e media.

**yl Alcohol.** Although ethyl alcohol in small amounts is produced ecies of several genera of molds, for example, *Aspergillus*, *Penicil-* *Clasterosporium*, *Helminthosporium geniculatum*, and *Mucor race-* it is produced commonly by species of the genus *Fusarium*. Yields nol comparable to those obtained from yeasts have been produced *ini* Bolley.

mechanism for the production of ethyl alcohol by fusaria has been by Anderson and his associates (1922, 1937); White and Willaman ; Nord (1939); Gould, Tytell, and Hughes (1939); and others. chanism for the production of ethanol by fusaria is believed to be to that for production by yeasts.

**erol.** Glycerol has been produced by a white species of *Asper-* y *A. wentii*, by a *Clasterosporium* species, by *H. geniculatum*, and *acemosus*.

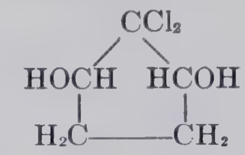
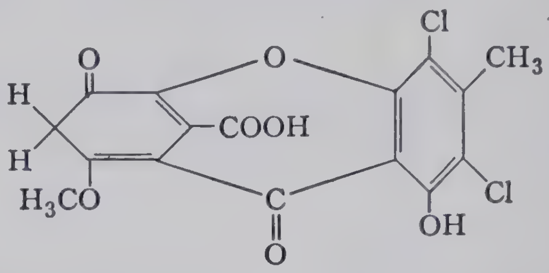
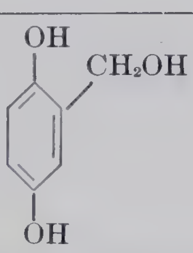
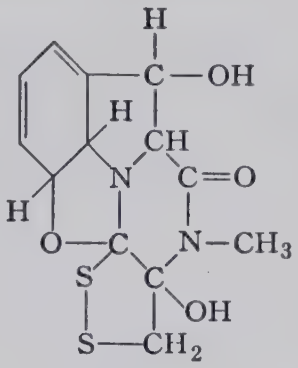
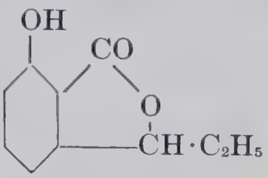
**hylglyoxal.** Sodium hexosediphosphate has been fermented by er with the production of methylglyoxal (Suthers and Walker,

**rine-containing Compounds.** Erdin [$C_{15}H_7O_6Cl_2(OCH_3)$] and [$C_{15}H_6O_5 \cdot Cl_2(OCH_3)_2$] are products of *A. terreus* Thom. Griseo- [$C_{17}H_{17}O_6Cl$] is a neutral, dextrorotatory crystalline substance d by *P. griseo-fulvum* Dierckx. Caldariomycin (see Table 201) iced by *Caldariomyces fumago*.

other products are formed by molds. The reader is referred to citations at the end of this chapter and to other sources for addi- nformation.

tensive investigations of Raistrick and associates, Robertson and

Table 201. Some Miscellaneous Mold Metabolic Produc

| Product | Structural formula | P |
|---|---|---|
| Caldariomycin $(C_5H_8O_2Cl_2)$ |  | Cald fu |
| Erdin |  | Asper |
| Gentisyl alcohol (2:5-dihydroxy-benzyl alcohol) |  | Penicil lum |
| Gliotoxin $(C_{13}H_{14}O_4N_2S_2)$ |  | A. fumi<br>P. cine<br>P. obsc<br>P. Terl<br>Trichod viride |
| Mellein (a lactone of 6-hydroxy-2-α-hydroxy-propylbenzoic acid) $(C_{10}H_{10}O_3)$ |  | A. melle<br>A. ochra |

collaborators, Birkinshaw and fellow workers, and Bu'Lock ar ates are recommended as fruitful sources of information. Brack covered many aspects of the biochemistry of molds in his treatis

## MOLDS AND ARSENIC COMPOUNDS

Sixty or more years ago the arsenic compounds present in the of some old wallpapers and in some plasters were occasionally of severe poisoning and even of fatalities. A fatal case of arseni ing of this nature occurred in England as recently as 1931 (C 1935).

number of theories have been proposed to explain the cause of the
ning. One of the earliest was that particles of the arsenic-containing
ent from the paper were inhaled. In 1839, Gmelin suggested that a
ile arsenic compound was responsible, since an odor of garlic
nearly always associated with the rooms where poisonings oc-
d. These rooms were commonly damp and moldy. Cacodyl oxide
$_3)_2\cdot As\cdot O\cdot As\cdot (CH_3)_2]$ was suggested by Basedow (1846) as being
ıbstance concerned. One year later, Martin proposed that the gas
be arsine ($AsH_3$). The subject was carefully studied by Gosio
). Gosio exposed to the air potato mashes that contained arsenious
Molds produced an odor of garlic from the potato mashes. One
which was named *Penicillium brevicaule* by Gosio, was particularly
in this respect. Other molds concerned were *Aspergillus glaucus,*
*ens,* and *Mucor mucedo.* The gas produced by these molds from
ous oxide, frequently referred to as "Gosio-gas," was shown by
nger and his associates (1933) to be trimethylarsine [$(CH_3)_3\cdot As$].
m and Raper (1932) have shown that *A. fischeri, A. sydowi,* and
organisms isolated from the soil may produce volatile substances
rsenic compounds.

llenger and his associates have shown that several organic com-
s of arsenic may be formed by certain molds. For example, methyl-
larsine is produced from diethylarsonic acid [$(CH_3CH_2)_2AsO\cdot OH$];
ıyl-*n*-propylarsine from *n*-propylarsonic acid; and dimethylallylar-
$(CH_3)_2As\cdot CH_2CH{:}CH_2$] from allylarsonic acid [$CH_2{:}CH\cdot CH_2$-
$H)_2$] by the action of *P. brevicaule* Saccardo, grown on sterile
rumbs.

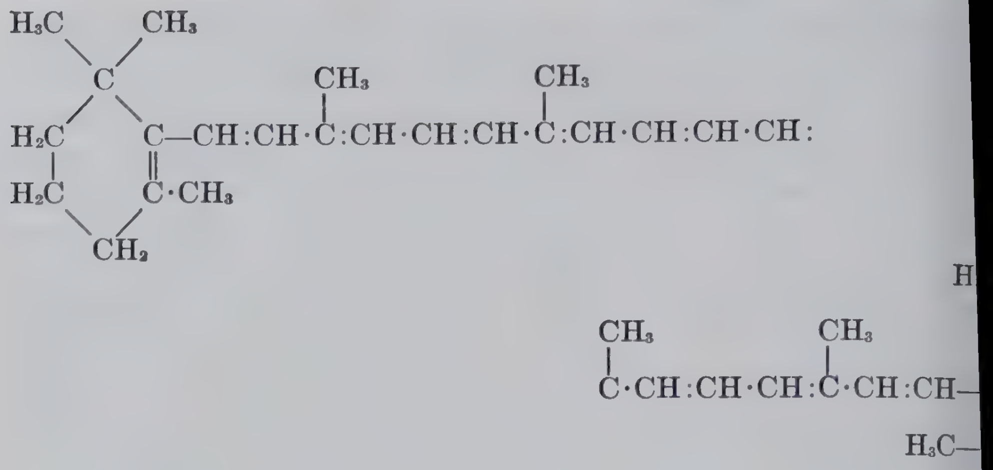
d alkylmethylarsines [$AsR(CH_3)_2$ and $AsR_2\cdot CH_3$] are obtained
yl-arsonic and dialkyl-arsonic acids ($R\cdot AsO_3H_2$ and $R_2AsO\cdot OH$)
ead inoculated with *P. brevicaule.* Dimethyl selenide is produced
dium selenate and selenite.

ther examples of methylating action, consult the papers by Chal-
nd his coworkers.

**tative Test for Arsenic.** Gosio (Challenger, 1935) developed an
ly sensitive test for the detection of arsenic. The material sus-
f containing arsenic was extracted with water or dilute acid. The
was concentrated by evaporation of some of the water. Some of
entrated extract was then added to a slice of potato that had been
ly sterilized and inoculated with a culture of *P. brevicaule,* and
ium was incubated at 25 to 30°C. An odor of garlic would develop
pound of arsenic was present. This test is said to be more sensitive
Marsh test and to be able to detect as small a quantity as
1 g. of arsenious oxide in 1 g. of substance.

## BETA-CAROTENE

tene, which is violet red in color in the pure state, is one of a group
nes and is known as provitamin A. It has the structural formula
page 698:



β-carotene occurs naturally in carrots, sweet potatoes, gre butter, egg yolks, fish oils, and certain other sources. It is pro species of the genera *Choanephora* and *Blakeslea* of the fam anephoraceae (class Phycomycetes) and other fungi; by yeasts *Rhodotorula flava, R. gracilis,* and *R. sannieli;* and by some s bacteria, algae, and lichens. Much β-carotene is chemically syn It is also produced economically by microbiological means on trial scale.

Schopfer and Jung (1935) reported that *Phycomyces blak* produced 0.09 to 0.1 per cent carotene (believed to be β-carot closely related carotenoid) when grown in a medium which c 0.4 per cent asparagine as the nitrogen source. Barnett, Lilly, an (1956) found that plus and minus strains of *C. cucurbitarum* yields of 960 μg. per g. β-carotene in the dried mycelium. Ciegler and Anderson (1958*a, b*) obtained yields of nearly 0.1 per cent wh plus and minus strains of *B. trispora.*

**Production by B. trispora.** The production of carotenoids and minus strains of *B. trispora* has been studied by Hessel Anderson (1957), Anderson et al. (1957), and Ciegler, Arnold, an son (1958*a, b*).

Cultures of Choanephoraceae may be grown at 25 to 28°C. o dextrose agar. Since stock cultures die rather rapidly at low temp they should not be stored at refrigeration temperatures. Stock may be maintained on slants, and transfers may be made satis from these until such time as the agar is nearly dried out.

Information relating to the determination of reactions of plus ar strains of the Choanephoraceae has been published by Hesselt Benjamin (1957).

Studies concerning carotene production by opposite mating grown singly and in pairs in a liquid synthetic mucor medium c in shake flasks, were made by Hesseltine and Anderson (195 medium contained 40 g. glucose, 2 g. asparagine, 0.5 g. $KH_2PO_4$ $MgSO_4$, 0.5 mg. thiamin, and 1 liter of distilled water. It was dis in 100-ml. portions in 500-ml. flasks, sterilized, cooled, inoculat ions of the vegetative mycelium, and incubated for 72 hr. at 28°C. on tary shaker. The yields of β-carotene produced by mated strains much higher than those by either parent alone.

a laboratory basis, good results have been obtained by Anderson associates with a production medium containing the following ingre-
s:

| | |
|---|---|
| Acid-hydrolyzed whole corn | 7.5% |
| Acid-hydrolyzed casein | 0.2% |
| $KH_2PO_4$ | 0.05% |
| Thiamin HCl | 1.0 mg. per liter |
| Nonionic detergent | 0.6–0.12% |
| Vegetable oils | 4.0% |
| β-Ionone | 0.1% |

pH of the medium is adjusted to 6.2 with NaOH before its steriliza- The β-ionone is added in an aseptic manner 48 hr. after the initial ation of the medium.

molds for use in inoculating the foregoing medium may be grown edium of the same composition, except that the oil, nonionic deter- and β-ionone should be omitted. The plus and minus strains are in separate flasks for 2 days. A suitable amount of inoculum for oduction medium is 5 per cent of each strain.

gler, Arnold, and Anderson (1958*a*) studied the effect of various on β-carotene production by *B. trispora*. They found that unhydro- grains, with the exception of corn, generally produced lower yields otene. When soybean meal extracted with hexane was used in f grain, about 8 mg. of carotene per gram of dry fermentation solids btained. Corn-steep liquor added to hydrolyzed corn did not the yields.

er et al. (1958*b*) also studied the influence of oils and fatty acids roduction of β-carotene by microorganisms. They found that the of various natural oils, fats, or waxes to grain-type media caused d production of β-carotene and that choice white grease gave the yields of carotene (8 to 9 mg. per g. of dry fermentation solids), d being proportional to the amount of grease added up to 4 per a result of studying the effect of various fatty acids on carotene ion, they concluded that optimum yields were obtained as a result ddition of either oleic acid or a solution of the mixed fatty acids fflower (yields of 6 mg. and 8.8 mg. of carotene per gram of dry ere obtained, respectively). The yields of mycelium obtained with ids were generally lower than those obtained with fats or oils.

**ction by Species of the Genus Rhodotorula.** Production of ne by *R. flava*, *R. gracilis*, and *R. pallida* has been studied by nd Clark (1958). Yields of β-carotene ranging from 5.2 to per g. of dry cells were obtained in cultures grown in a non- medium.

## References on the Biochemistry of Molds

### I. Studies by Raistrick and His Associates [1]

1. Raistrick, H., and W. Rintoul: I. Introductory, p. 2.
2. Birkinshaw, J. H., and H. Raistrick: II. Quantitative Methods and T of Investigation of the Products of Metabolism of Micro-organisms,
3. ———, J. H. V. Charles, H. Raistrick, and J. A. R. Stoyle: III. Qu Examination by the Carbon Balance Sheet Method of the Types of Formed from Glucose by Species of *Aspergillus,* p. 27.
4. ———, ———, A. C. Hetherington, and H. Raistrick: IV. Quantitativ nation by the Carbon Balance Sheet Method of the Types of Product from Glucose by Species of *Penicillium* (including *Citromyces*), appendix by C. Thom, p. 55.
5. ———, ———, H. Raistrick and J. A. R. Stoyle: V. Quantitative Exa by the Carbon Balance Sheet Method of the Types of Products form Glucose by Species of *Fusarium,* p. 93.
6. ———, ———, A. C. Hetherington, and H. Raistrick: VI. Quantitative nation by the Carbon Balance Sheet Method of the Types of Products from Glucose by Miscellaneous Species of Fungi, p. 99.
7. ———, ———, C. H. Lilly, and H. Raistrick: VII. On Kojic Acid (5-H 2-hydroxy-methyl-$\gamma$-pyrone), p. 127.
8. ——— and H. Raistrick: VIII. The Estimation of Kojic Acid, p. 139.
9. ———, J. H. V. Charles, A. C. Hetherington, and H. Raistrick: IX. Production of Mannitol from Glucose by Species of *Aspergillus,* p. 153.
10. Raistrick, H., and W. Young: X. The Estimation of Mannitol in Ferme Solution, p. 173.
11. Hetherington, A. C., and H. Raistrick: XI. On Citromycetin, a New Colouring Matter Produced from Glucose by Species of *Citromyces,* p.
12. Birkinshaw, J. H., and H. Raistrick: XII. On a New Methoxy-dihy toluquinone Produced from Glucose by Species of *Penicillium* of the *P. losum* Series, p. 245.
13. Raistrick, H., and M. L. Rintoul: XIII. On a New Type of Mucilaginous rial, Luteic Acid, Produced from Glucose by *Penicillium luteum* Zukal,
14. Hetherington, A. C., and H. Raistrick: XIV. On the Production and Ch Constitution of a New Yellow Colouring Matter, Citrinin, Produced fro cose by *Penicillium citrinum* Thom, p. 269.
15. Coyne, F. P., H. Raistrick, and R. Robinson: XV. The Molecular Struct Citrinin, p. 297.
16. Clutterbuck, P. W., H. Raistrick, and M. L. Rintoul: XVI. On the Prod from Glucose by *Penicillium spiculisporum* Lehman of a New Polybasic Acid, $C_{17}H_{28}O_6$ (the Lactone of $\gamma$-Hydroxy-$\beta\delta$-dicarboxy-pentadecoic p. 301.
17. Birkinshaw, J. H., and H. Raistrick: XVII. The Products of Glucose Meta Formed by Various Species of Fungi (*Helminthosporium, Clasterosp* etc.), p. 331.
18. ———, J. H. V. Charles, and H. Raistrick: XVIII. Biochemical Characte of Species of *Penicillium* Responsible for the Rot of Citrus Fruits, p. 3
19. Anslow, W. H., and R. Raistrick: XIX. 6-Hydroxy-2-methylbenzoic A Product of the Metabolism of Glucose by *Penicillium griseo-fulvum* Di *Biochem. Jour.,* **25**: 39 (1931).
20. Coyne, F. P., and H. Raistrick: XX. On the Production of Mannitol Hexoses and Pentoses by a White Species of *Aspergillus, Biochem. Jou* 1513 (1931).

---

[1] The first 18 of these studies were published in the *Philosophical Transacti the Royal Society of London, Series B,* **220**: 1–367 (1931), under the title "Stu the Biochemistry of Micro-organisms," by Harold Raistrick and his associates.

irkinshaw, J. H., J. H. V. Charles, and P. W. Clutterbuck: XXI. Quantitative Examination by the Carbon Balance Sheet Method of the Types of Products Formed from Glucose by Species of Bacteria, *Biochem. Jour.*, **25**: 1522 (1931).

——, R. K. Callow, and C. F. Fischmann. XXII. The Isolation and Characterisation of Ergosterol from *Penicillium puberulum* Bainier Grown on a Synthetic Medium with Glucose as a Sole Source of Carbon, *Biochem. Jour.*, **25**: 1977 (1931).

—— and H. Raistrick· XXIII. Puberulic Acid $C_8H_6O_6$ and an Acid $C_8H_4O_6$, New Products of the Metabolism of Glucose by *Penicillium puberulum* Bainier ind *Penicillium aurantio-virens* Biourge, with an Appendix on Certain Dihydroxybenzene-dicarboxylic Acids, *Biochem. Jour.*, **26**: 441 (1932).

utterbuck, P. W., A. E. Oxford, H. Raistrick, and G. Smith: XXIV. The Metabolic Products of the *Penicillium brevi-compactum* Series, *Biochem. Jour.*, **26**: 1441 (1932).

ford, A. E., and H. Raistrick: XXV. 3:5-Dihydroxyphthalic Acid, a New Product of the Metabolism of Glucose by *Penicillium brevi-compactum* Dierckx and Related Species, *Biochem. Jour.*, **26**: 1902 (1932).

utterbuck, P. W., R. Lovell, and H. Raistrick: XXVI. The Formation from Glucose by Members of the *Penicillium chrysogenum* Series of a Pigment, n Alkali Soluble Protein, and Penicillin—the Antibacterial Substance of Fleming, *Biochem. Jour.*, **26**: 1907 (1932).

kinshaw, J. H., and H. Raistrick: XXVII. The Production of Luteic Acid rom Various Sources of Carbon by *Penicillium luteum* Zukal, *Biochem. Jour.*, 7: 370 (1933).

arles, J. H. V., H. Raistrick, R. Robinson, and A. R. Todd: XXVIII. Helminhosporin and Hydroxyisohelminthosporin, Metabolic Products of the Plant Pathogen *Helminthosporium gramineum* Rabenhorst, *Biochem. Jour.*, **27**: 499 1933).

strick, H., and P. Simonart: XXIX. 2:5-Dihydroxybenzoic Acid (Gentisic cid), a New Product of the Metabolism of Glucose by *Penicillium griseoulvum* Dierckx, *Biochem. Jour.*, **27**: 628 (1933).

ord, A. E., and H. Raistrick: XXX. The Molecular Constitution of the etabolic Products of *Penicillium brevi-compactum* Dierckx and Related pecies. I. The Acids $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, and $C_{10}H_{10}O_7$, *Biochem. Jour.*, **27**: 634 933).

terbuck, P. W., and H. Raistrick: XXXI. The Molecular Constitution of e Metabolic Products of *Penicillium brevi-compactum* Dierckx and Related pecies. II. Mycophenolic Acid, *Biochem. Jour.*, **27**: 654 (1933).

trick, H., R. Robinson, and A. R. Todd: XXXII. Cynodontin (1:4:5:8-trahydroxy-2-Methylanthraquinone), a Metabolic Product of *Helminthorium cynodontis* Marignoni and *Helminthosporium euchlaenae* Zimmerınn, *Biochem. Jour.*, **27**: 1170 (1933).

rd, A. E., and H. Raistrick: XXXIII. The Mycelial Constituents of *Penilium brevi-compactum* Dierckx and Related Species. I. Ergosterol Palmite, *Biochem. Jour.*, **27**: 1176 (1933).

— and ——: XXXIV. A Note on the Mechanism of the Production of enolic Acids from Glucose by *Penicillium brevi-compactum* Dierckx, *Biom. Jour.*, **27**: 1473 (1933).

trick, H., and G. Smith: XXXV. The Metabolic Products of *Byssochlamys va* Olliver and Smith, *Biochem. Jour.*, **27**: 1814 (1933).

terbuck, P. W., W. N. Haworth, H. Raistrick, G. Smith, and M. Stacey: XXVI. The Metabolic Products of *Penicillium charlesii* G. Smith, *Biochem. r.*, **28**: 94 (1934).

rick, H., R. Robinson, and A. R. Todd: XXXVII. (*a*) On the Production Hydroxyanthraquinones by Species of *Helminthosporium*. (*b*) Isolation of tisporin, a New Metabolic Product of *Helminthosporium tritici-vulgaris* sikado. (*c*) The Molecular Constitution of Catenarin, *Biochem. Jour.*, **28**: (1934).

nshaw, J. H., and H. Raistrick: XXXVIII. The Metabolic Products of

*Penicillium minio-luteum* Dierckx. Minioluteic Acid, *Biochem.* (1934).

39. Oxford, A. E., and H. Raistrick: XXXIX. The Metabolic Prod *cillium crateriforme* Gilman and Abbott, *Biochem. Jour.,* **28**: 132

40. Gould, B. S., and H. Raistrick: XL. The Crystalline Pigments of *Aspergillus glaucus* Series, *Biochem. Jour.,* **28**: 1640 (1934).

41. Clutterbuck, P. W., H. Raistrick, and F. Reuter: XLI. The Metab of *Penicillium charlesii* G. Smith. II. The Molecular Constituti and Carolinic Acids, *Biochem. Jour.,* **29**: 300 (1935).

42. Raistrick, H., and G. Smith: XLII. The Metabolic Products o *terreus* Thom. A New Mould Metabolic Product—Terrein, *Bio* **29**: 606 (1935).

43. Clutterbuck, P. W., H. Raistrick, and F. Reuter: XLIII. The Met ucts of *Penicillium charlesii* G. Smith. III. The Molecular Co Carlic and Carlosic Acids, *Biochem. Jour.,* **29**: 871 (1935).

44. Oxford, A. E., H. Raistrick, and P. Simonart: XLIV. Fulvic Acid, a talline Yel'ow Pigment, a Metabolic Product of *P. griseo-fulvu P. flexuosum* Dale and *P. brefeldianum* Dodge, *Biochem. Jour* (1935).

45. Clutterbuck, P. W., H. Raistrick, and F. Reuter: XLV. The Metabol of *Penicillium charlesii* G. Smith. IV. *l*-γ-Methyltetronic Acid, wit tions on the Formation and Structure of Ramigenic and Vertic *Biochem. Jour.,* **29**: 1300 (1935).

46. Oxford, A. E., and H. Raistrick: XLVI. *i*-Erythritol, a New Metabol of *Penicillium brevi-compactum* Dierckx and *P. cyclopium* Westling *Jour.,* **29**: 1599 (1935).

47. Anderson, C. G., and H. Raistrick: XLVII. The Production of Polys by *Penicillium luteum* Zukal, *Biochem. Jour.,* **30**: 16 (1936).

48. Birkinshaw, J. H., A. E. Oxford, and H. Raistrick: XLVIII. Penicil Metabolic Product of *Penicillium puberulum* Bainier and *P. cyclopi* ling, *Biochem. Jour.,* **30**: 394 (1936).

49. ——— and H. Raistrick: XLIX. Palitantin, $C_{14}H_{22}O_4$, a Hitherto U Metabolic Product of *Penicillium palitans* Westling, *Biochem. Jou* (1936).

50. Raistrick, H., R. Robinson, and E. White: L. Ravenelin (3-Met Trihydroxyxanthone), a New Metabolic Product of *Helminthospor nelii* Curtis and of *H. turcicum* Passerini, *Biochem. Jour.,* **30**: 1303 (1

51. ——— and G. Smith: LI. The Metabolic Products of *Aspergillus terre* II. Two New Chlorine-containing Mould Metabolic Products, Ge Erdin, *Biochem. Jour.,* **30**: 1315 (1936).

52. Birkinshaw, J. H., and H. Raistrick: LII. Isolation, Properties and tion of Terrestric Acid (Ethylcarolic Acid), a Metabolic Product of *P terrestre* Jensen, *Biochem. Jour.,* **30**: 2194 (1936).

53. Ashley, J. N., B. C. Hobbs, and H. Raistrick: LIII. The Crystalline Matters of *Fusarium culmorum* (W. G. Smith) Sacc. and Relate *Biochem. Jour.,* **31**: 385 (1937).

54. Clutterbuck, P. W., H. Raistrick, and F. Reuter: LIV. The Molecula tution of Terrein, a Metabolic Product of *Aspergillus terreus* Th *chem. Jour.,* **31**: 987 (1937).

55. ———, W. Koerber, and H. Raistrick: LV. The Molecular Consti Geodin and Erdin, Two Chlorine-containing Metabolic Products o *gillus terreus* Thom. I. The Constitutional Relationship of Geo Erdin, *Biochem. Jour.,* **31**: 1089 (1937).

56. Ashley, J. N., and H. Raistrick: LVI. Luteoleersin and Alboleersin; Products of *Helminthosporium leersii* Atkinson. A Possible Oxidati tion System, *Biochem. Jour.,* **32**: 449 (1938).

57. Anslow, W. K., and H. Raistrick: LVII. Fumigation (3-Hydroxy-4- 2:5-Toluquinone) and Spinulosin (3:6-Dihydroxy-4-Methoxy-2:5-

none) Metabolic Products Respectively of *Aspergillus fumigatus* Fresenius and *Penicillium spinulosum* Thom, *Biochem. Jour.*, **32**: 687 (1938).

—— and ———: LVIII. Synthesis of Spinulosin (3:6-Dihydroxy-4-Methoxy-2:5-Toluquinone) a Metabolic Product of *Penicillium spinulosum* Thom, *Biochem. Jour.*, **32**: 803 (1938).

aistrick, H., and W. K. Anslow: LIX. Spinulosin (3:6-Dihydroxy-4-Methoxy-2:5-Toluquinone) a Metabolic Product of a Strain of *Aspergillus fumigatus* Fresenius, *Biochem. Jour.*, **32**: 2288 (1938).

xford, A. E., H. Raistrick, and P. Simonart: LX. Griseofulvin $C_{17}H_{17}O_6Cl$, a Metabolic Product of *Penicillium griseo-fulvum* Dierckx, *Biochem. Jour.*, **33**: 240 (1939).

alam, C. T., P. W. Clutterbuck, A. E. Oxford, and H. Raistrick: LXI. The Molecular Constitution of Geodin and Erdin, Two Chlorine-containing Metabolic Products of *Aspergillus terreus* Thom. II. Dihydrogeodin and Dihydroerdin Ethers, *Biochem. Jour.*, **33**: 579 (1939).

hley, J. N., H. Raistrick, and T. Richards: LXII. The Crystalline Colouring Matters of Species in the *Aspergillus glaucus* Series, II. *Biochem. Jour.*, **33**: 1291 (1939).

lam, C. T., A. E. Oxford, and H. Raistrick: LXIII. Itaconic Acid, a Metabolic Product of a Strain of *Aspergillus terreus* Thom, *Biochem. Jour.*, **33**: 1488 (1939).

slow, W. K., J. Breen, and H. Raistrick: 64. Emodic Acid (4:5:7-Trihydroxy-anthraquinone-2-carboxylic Acid) and ω-Hydroxy-Emodin (4:5:7-Trihy-lroxy-2-(hydroxymethyl)-anthraquinone), Metabolic Products of a Strain of *enicillium cyclopium* Westling, *Biochem. Jour.*, **34**(2): 159–168 (1940).

itterbuck, P. W., S. L. Mukhopadhyay, A. E. Oxford, and H. Raistrick: 65 A). A Survey of Chlorine Metabolism by Moulds, (B) Caldariomycin, $_5H_8O_2Cl_2$, A Metabolic Product of *Caldariomyces fumago* Woronichin, *Bio-hem. Jour.*, **34**(5): 664–677 (1940).

ford, A. E., and H. Raistrick: 66. Penicilliopsin, the Colouring Matter of *Peni-illiopsis clavariaeformis* Solms-Laubach, *Biochem. Jour.*, **34**(6): 790–803 (1940).

low, W. K., and H. Raistrick: 67. The Molecular Constitutions of Catenarin id Erythroglaucin, Metabolic Products Respectively of *Helminthosporium tenarium* Drechsler and of Species in the *Aspergillus glaucus* Series, *Biochem. ur.*, **34**(7): 1124–1133 (1940).

— and ———: 68. Synthesis of Cynodontin (1:4:5:8-tetrahydroxy-2-ethylanthraquinone), A Metabolic Product of Species of *Helminthosporium*, *ochem. Jour.*, **34**(12): 1546–1548 (1940).

— and ———: 69. Synthesis of Catenarin (1:4:5:7-tetrahydroxy-2-methyl-thraquinone), a Metabolic Product of Species of *Helminthosporium*, *Bio-em. Jour.*, **35**(8 and 9): 1006–1010 (1941).

inshaw, J. H., A. R. Chambers, and H. Raistrick: 70. Stipitatic Acid, $H_6O_5$, a Metabolic Product of *Penicillium stipitatum* Thom, *Biochem. Jour.*, (1 and 2): 242–251 (1942).

—, H. Raistrick, and G. Smith: 71. Fumaryl-*dl*-alanine (Fumaromono-*dl*-mide), a Metabolic Product of *Penicillium resticulosum* sp. nov., *Biochem. our.*, **36**(10, 11, and 12): 829–835 (1942).

—, A. Bracken, and H. Raistrick: 72. Gentisyl Alcohol (2:5-dihydroxy-nzyl alcohol), a Metabolic Product of *Penicillium patulum* Bainier, *Bio-m. Jour.*, **37**(6): 726–728 (1943).

—, ———, and ———: 73. Metabolic Products of *Aspergillus fumigatus* senius, *Biochem. Jour.*, **39**(1): 70–72 (1945).

n, C. T., P. W. Clutterbuck, A. E. Oxford, and H. Raistrick: 74. The lecular Constitution of Geodin and Erdin, Two Chlorine-containing Meta-ic Products of *Aspergillus terreus* Thom. Part 3. Possible Structural mulae for Geodin and Erdin, *Biochem. Jour.*, **41**: 458–463 (1947).

n, A., and H. Raistrick: 75. Dehydrocarolic Acid, a Metabolic Product *Penicillium cinerascens* Biourge, *Biochem. Jour.*, **41**: 569–575 (1947).

, A. E., and H. Raistrick: 76. Mycelianamide, $C_{22}H_{28}O_5N_2$, a Metabolic

Product of *Penicillium griseo-fulvum* Dierckx. Part I. Preparation,
and Breakdown Products, *Biochem. Jour.,* **42**: 323–329 (1948).

77. Raistrick, H., and J. M. Vincent: 77. A Survey of Fungal Metabolis
ganic Sulfates, *Biochem. Jour.,* **43**: 90–99 (1948).

78. Birkinshaw, J. H., A. Bracken, E. N. Morgan, and H. Raistrick: 78. T
ular Constitution of Mycophenolic Acid, a Metabolic Product of *P*
*brevi-compactum* Dierckx. Part II. Possible Structural Formulae
phenolic Acid, *Biochem. Jour.,* **43**: 216–223 (1948).

79. Michael, S. E.: 79. Fuscin, a Metabolic Product of *Oidiodendro*
Robak. Part I. Preparation, Properties and Antibacterial Activity,
*Jour.,* **43**: 528–533 (1948).

80. Howard, B. H., and H. Raistrick: 80. The Colouring Matters of *P*
*islandicum* Sopp. Part I. 1:4:5-Trihydroxy-2-methylanthraquinone,
*Jour.,* **44**: 227–233 (1949).

81. ——— and ———: 81. The Colouring Matters of *Penicillium islandic*
Part II. Chrysophanic Acid, 4:5-Dihydroxy-2-methylanthraquinone,
*Jour.,* **46**: 49–53 (1950).

82. Raistrick, H., and C. E. Stickings: 82. Ustic Acid, a Metabolic Pr
*Aspergillus ustus* (Bainier) Thom & Church, *Biochem. Jour.,* **48**: 53–6

83. Birkinshaw, J. H., A. Bracken, S. E. Michael, and H. Raistrick: 83.
Metabolic Product of *Oidiodendron fuscum* Robak. Part 2. Derivat
Degradation Products, *Biochem. Jour.,* **48**: 64–74 (1951).

84. Raistrick, H., and J. Ziffer: 84. The Colouring Matters of *Penicillium*
*vensis* Laxa. Part 1. Nalgiovensin and Nalgiolaxin. Isolation, Derivat
Partial Structures, *Biochem. Jour.,* **49**: 563–574 (1951).

85. Birkinshaw, J. H., H. Raistrick, D. J. Ross, and C. E. Stickings: 85. C
and Cyclopaldic Acids, Metabolic Products of *Penicillium cyclopium* W
*Biochem. Jour.,* **50**: 610–628 (1952).

86. ———, ———, and ———: 86. The Molecular Constitution of Myco
Acid, a Metabolic Product of *Penicillium brevi-compactum* Dierckx.
phenolic Acid, *Biochem. Jour.,* **50**: 630–634 (1952).

87. Raistrick, H., and D. J. Ross: 87. Dihydrogladiolic Acid, a Metabolic
of *Penicillium gladioli* Machacek, *Biochem. Jour.,* **50**: 635–647 (1952).

88. Birkinshaw, J. H.: 88. Palitantin. Part 2. Further Derivatives and Deg
Products, *Biochem. Jour.,* **51**: 271–276 (1952).

89. ———: 89. Metabolic Products of *Penicillium multicolor* G.-M. and
special reference to Sclerotiorin, *Biochem. Jour.,* **52**: 283–288 (1952).

90. Raistrick, H., C. E. Stickings, and R. Thomas: 90. Alternariol and Alt
Monomethyl Ether, Metabolic Products of *Alternaria tenuis, Biochem*
**55**: 421–433 (1953).

91. Howard, B. H., and H. Raistrick: 91. The Colouring Matters of *Pen*
*islandicum* Sopp. Part 3. Skyrin and Flavoskyrin, *Biochem. Jour.,* **56**
(1954).

92. ——— and ———: 92. The Colouring Matters of *Penicillium islandicum*
Part 4. Iridoskyrin, Rubroskyrin and Erythroskyrin, *Biochem. Jour.,* **5**
222 (1954).

93. Bracken, A., A. Pocker, and H. Raistrick: 93. Cyclopenin, A Nitrogen-con
Metabolic Product of *Penicillium cyclopium* Westling, *Biochem. Jou*
587–595 (1954).

94. Howard, B. H., and H. Raistrick: 94. The Colouring Matters of Species
*Aspergillus nidulans* Group. Part 1. Asperthecin, a Crystalline Co
Matter of *Aspergillus quadrilineatus* Thom & Raper, *Biochem. Jour.,* **5**
486 (1955).

95. Breen, J., J. C. Dacre, H. Raistrick, and G. Smith: 95. Rugulosin, a cry
Colouring Matter of *Penicillium rugulosum* Thom, *Biochem. Jour.,* **6**
626 (1955).

96. Galarraga, J. A., K. G. Neill, and H. Raistrick: 96. The Colouring Mat
*Penicillium herquei* Bainier & Sartory, *Biochem. Jour.,* **61**: 456–464 (19

97. Raistrick, H., and P. Rudman: 97. Flavipin, a Crystalline Metabolite of

*gillus flavipes* (Bainier & Sartory) Thom & Church and *Aspergillus terreus* Thom, *Biochem. Jour.*, **63**: 395–406 (1956).

'eelakantan, S., A. Pocker, and H. Raistrick: 98. Tritisporin, An Anthraquinone Colouring Matter of *Helminthosporium tritici-vulgaris* Nisikado: Structure and Synthesis. *Biochem. Jour.*, **64**: 464–469 (1956).

irkinshaw, J. H., and I. M. M. Hammady: 99. Metabolic Products of *Aspergillus versicolor* (Vuillemin) Tiraboschi, *Biochem. Jour.*, **65**: 162–166 (1957).

eill, K. G., and H. Raistrick: 100. Metabolites of *Penicillium atrovenetum* G. Smith. Part 1. Atrovenetin, a New Crystalline Colouring Matter, *Biochem. Jour.*, **65**: 166–176 (1957).

## II. Studies by A. Robertson and Associates

acKenzie, J. B. D., and A. Robertson: Part I. The Synthesis of 6-Hydroxy-3:4-dimethoxyphthalic Acid, *Jour. Chem. Soc.*, 1949, pp. 497–499.

ones, G. H., J. B. D. MacKenzie, A. Robertson, and W. B. Whalley: Part II. Derivatives of 3:4-Dimethoxyphenol, *Jour. Chem. Soc.*, 1949, pp. 562–569.

obertson, A., and W. B. Whalley: Part III. The Degradation of 0-Dimethylcitromycin and the Structure of Citromycetin, *Jour. Chem. Soc.*, 1949, pp. 848–859.

own, J. P., N. J. Cartwright, A. Robertson, and W. B. Whalley: Part IV. The Constitution of the Phenol, $C_{11}H_{16}O_3$, from Citrinin, *Jour. Chem. Soc.*, 1949, pp. 859–867.

——, A. Robertson, W. B. Whalley, and N. J. Cartwright: Part V. The Constitution of Citrinin, *Jour. Chem. Soc.*, 1949, pp. 867–879.

obertson, A., W. R. Smithies, and E. Tittensor: Part VI. Rosenonolactone from *Trichothecium roseum* Link, *Jour. Chem. Soc.*, 1949, pp. 879–884.

rtwright, N. J., A. Robertson, and W. B. Whalley: Part VII. Syntheses of itrinin and Dihydrocitrinin, *Jour. Chem. Soc.*, 1949, pp. 1563–1567.

vill, G. W. K., A. Robertson, and W. B. Whalley: Part VIII. The Oxidation f Methylene Groups in Compounds Analogous to 0-Dimethylcitromycin, *our. Chem. Soc.*, 1949, pp. 1567–1570.

n, F. M., A. Robertson, and W. B. Whalley: Part IX. 3:4-Dihydrocoumarins, *our. Chem. Soc.*, 1950, pp. 895–902.

cock, G. G., F. M. Dean, A. Robertson, and W. B. Whalley: Part X. The nthesis of 4-Hydroxy-3-acetylcoumarins, *Jour. Chem. Soc.*, 1950, pp. 903–908.

ill, G. W. K., A. Robertson, and W. B. Whalley: Part XI. The Degradation 0-Dimethylcitromycinol and a Revised Structure for 0-Dimethylcitromycin, *ur. Chem. Soc.*, 1950, pp. 1031–1037.

Kenzie, J. B. D., A. Robertson, and W. B. Whalley: Part XII. The Synthe- of 6′:7′-Dimethoxy-2:4-dimethylchromeno (3′:4′–5:6) Pyranol Identical th a Derivative of 0-Dimethylcitromycin, *Jour. Chem. Soc.*, 1950, pp. 2965–71.

son, D. H., A. Robertson, and W. B. Whalley: Part XIII. Citrinin, *Jour. hem. Soc.*, 1950, pp. 2971–2975.

h, B. J., and A. Robertson: Part XIV. 2:4:5-Trihydroxyphenylglyoxylic cid from *Polyporus tumulosus* Cooke, *Jour. Chem. Soc.*, 1950, pp. 3380–3383.

ertson, A., W. B. Whalley, and J. Yates: Part XV. The Degradation of ethyl 0-Dimethylcitromycetin, *Jour. Chem. Soc.*, 1951, pp. 2013–2018.

oigne, R. M., J. S. E. Holker, B. J. Ralph, and A. Robertson: Part XVI. uricoic Acid, *Jour. Chem. Soc.*, 1951, pp. 2346–2352.

—, A. Robertson, and J. J. Simes: Part XVII. Dehydroeburicoic Acid, *Jour. em. Soc.*, 1951, pp. 1830–1837.

er, J. S. E., A. D. G. Powell, A. Robertson, J. J. Simes, and R. S. Wright: rt XVIII. The Cyclic System of Eburicoic Acid, *Jour. Chem. Soc.*, 1953, 2414–2422.

—, ——, ——, ——, ——, and R. M. Gascoigne: Part XIX. The ucture of Eburicoic Acid, *Jour. Chem. Soc.*, 1953, pp. 2422–2429.

son, D. H., A. Robertson, and W. B. Whalley: Part XX. Metabolites of *etomium indicum* Corda, *Jour. Chem. Soc.*, 1953, pp. 2429–2434.

121. Lund, N. A., A. Robertson, and W. B. Whalley: Part XXI. Asperx a Preliminary Examination of Aspergillin, *Jour. Chem. Soc.,* 195 2439.

122. Dean, F. M., J. C. Roberts, and A. Robertson: Part XXII. Niduli nidulin ("Ustin"): Chlorine-containing Products of *Aspergillus nid Chem. Soc.,* 1954, pp. 1432–1439.

123. Cort, L. A., R. M. Gascoigne, J. S. E. Holker, B. J. Ralph, A. Rob J. J. H. Simes: Part XXIII. Tumulosic Acid, *Jour. Chem. Soc.,* 195 3721.

124. Dean, F. M., A. M. Osman, and A. Robertson: Part XXIV. The Fo Diquinones, *Jour. Chem. Soc.,* 1955, pp. 11–17.

125. Lloyd, G., A. Robertson, G. B. Sankey, and W. H. Whalley: P Oosporein, a Metabolite of *Chaetomium aureum* Chivers, *Jour. C* 1955, pp. 2163–2165.

126. Dean, F. M., A. D. T. Erni, and A. Robertson: Part XXVI. Dichloro *Jour. Chem. Soc.,* 1956, pp. 3545–3548.

### III. Studies by Birkinshaw and Associates

127. Birkinshaw, J. H., and W. P. K. Findlay: Biochemistry of the Wo Fungi. 1. Metabolic Products of *Lentinus lepideus* Fr., *Biochem.* 82–88 (1940).

128. ———, ———, and R. A. Webb: 2. A Study of the Acids Produced *ophora cerebella* Pers., *Biochem. Jour.,* **34**: 906–916 (1940).

129. ———, ———, and ———: 3. The Production of Methyl Mercaptan b *phyllum commune* Fr., *Biochem. Jour.,* **36**: 526–529 (1942).

130. ———, A. Bracken, and W. P. K. Findlay: 4. Metabolic Products of *suaveolens* (Linn.) Fr., *Biochem. Jour.,* **38**: 131–132 (1944).

131. ———, C. E. Stickings, and P. Tessier: 5. The Production of D- (L-Erythritol) by *Armillaria mellea* (Wahl) Quelet, *Biochem. Jour.,* 332 (1948).

132. ——— and E. N. Morgan: 6. Volatile Metabolic Products of Species *conidiophora, Biochem. Jour.,* **47**: 55–59 (1950).

133. ———, ———, and W. P. K. Findlay: 7. Metabolic Products of P *benzoinus* (Wahl.) Fr., *Biochem. Jour.,* **50**: 509–517 (1952).

### IV. Studies at the University of Tokyo (Series on Metabolic Products of

134. Shibata, S., O. Tanaka, and I. Kitagawa: V. The Structure of Skyrin, *Bull.,* 3(4): 278–283 (1955).

135. Tanaka, O., and C. Kaneko: VI. The Structure of Skyrin, *Pharm. Bul* 284–286 (1955).

136. Shibata, S., M. Takido, and T. Nakajima: VII. Paper Chromatograph Coloring Matters of *Penicillium islandicum* Sopp, *Pharm. Bull.,* 3(4 290 (1955).

137. ———, T. Murakimi, and M. Takido: IX. The Structure of Rugulosin Relation to the Structure of Flavoskyrin, *Pharm. Bull.,* **4**(4): 303–308

138. ——— and I. Kitagawa: X. The Structure of Rubroskyrin and Its Rela the Structure of Luteoskyrin, *Pharm. Bull.,* **4**(4): 309–313 (1956).

139. ———, T. Murakami, I. Kitagawa, and M. Takido: On the Fungal C Matters. The Structures of Rugulosin, Rubroskyrin, and Flavoskyri *Japan Acad.,* **32**(5): 356–360 (1956).

### V. Some Other Studies

140. Albericci, V. J., T. P. Curtin, and D. Reilly: Sclerotiose- a Polysaccharide bolic Product of *Penicillium sclerotiorum* Van Beyma, *Biochem. Jou* 243–246 (1943).

141. Anderson, A. K., E. L. Everett, and P. D. Adams: The Carbon Metabo *Fusarium oxysporum* on Glucose, *Jour. Agr. Research,* **46**: 473 (1933).

142. ——— and J. J. Willaman: *Proc. Soc. Exptl. Biol. Med.,* **20**: 108 (1922)

nderson, C. G., W. N. Haworth, H. Raistrick, and M. Stacey: Polysaccharides Synthesized by Micro-organisms. IV. The Molecular Constitution of Luteose, *Biochem. Jour.*, **33**: 272 (1939).

irkinshaw, J. H.: Biochemistry of the Lower Fungi, *Biol. Rev., Cambridge Phil. Soc.*, 12: 357 (1937).

——: *Brit. Mycol. Soc. Trans.*, **30**: 50 (1948).

racken, A.: "The Chemistry of Micro-organisms," Pitman Publishing Corporation, New York, 1955.

u'Lock, J. D.: The Constituents of the Higher Fungi. Part IV. A Quinone from *Polyporus fumosus, Jour. Chem. Soc.*, 1955, p. 575.

——: Acetylenic Compounds as Natural Products, *Quart. Revs. London*, 10(4): 371–394 (1956).

——, E. R. H. Jones, and P. R. Leeming: Chemistry of the Higher Fungi. Part V. The Structure of Nemotinic Acid and Nemotin, *Jour. Chem. Soc.*, 1955, pp. 4270–4276.

——, ——, and ——: Chemistry of the Higher Fungi. Part VII. Odyssic Acid and Odyssin, *Jour. Chem. Soc.*, No. 216, 1957, pp. 1097–1101.

——, ——, ——, and J. M. Thompson: Chemistry of the Higher Fungi. Part VI. Isomerization Reactions of Naturally Occurring Allenes, *Jour. Chem. Soc.*, No. 733, 1956, pp. 3767–3771.

——, ——, G. H. Mansfield, J. W. Thompson, and M. C. Whiting: The Structures of Two Polyacetylenic Antibiotics, *Chemistry & Industry*, 1954, pp. 990–991.

——, ——, and W. B. Turner: Chemistry of the Higher Fungi. Part VIII. A Series of Acetylenic Compounds from *Polyporus anthracophilus, Jour. Chem. Soc.*, No. 308, 1957, pp. 1607–1617.

—— and E. F. Leadbeater: The Production of Polyacetylenic Compounds by asidiomycetes: Glucose Conversion, *Biochem. Jour.*, **62**(3): 476–480 (1956).

allenger, F.: The Biological Methylation of Compounds of Arsenic and elenium, *Jour. Soc. Chem. Ind.*, **54**: 657–662 (1935).

— and L. Ellis: The Formation of Organo-metalloidal Compounds by icro-organisms. Part III. Methylated Alkyl- and Dialkyl-arsines, *Jour. hem. Soc.*, **87**: 396–400 (1935).

—, C. Higginbottom, and L. Ellis: The Formation of Organo-metalloidal ompounds by Micro-organisms. Part I. Trimethylarsine and Dimethylhylarsine, *Jour. Chem. Soc.*, 1933, p. 95.

— and L. Klein: The Formation of *l*-Malic Acid from Fumaric Acid by *spergillus niger, Jour. Chem. Soc.*, 1929, p. 1644.

— and A. A. Rawlings: The Formation of Organo-metalloidal Compounds Micro-organisms. Part IV. Dimethyl-*n*-propylarsine and Methylethyl-*n*-opylarsine, *Jour. Chem. Soc.*, **57**: 264–267 (1936).

terbuck, P. W.: Recent Developments in the Biochemistry of Moulds, *ur. Soc. Chem. Ind.*, **55**: 55T (1936).

in, T., G. Fitzgerald, and J. Reilly: Production of Phoenicine on Synthetic edia 1. *Penicillium phoeniceum* Van Beyma 2. *Penicillium rubrum* rasberger-Stoll, *Biochem. Jour.*, **34**: 1605–1610 (1940).

— and J. Reilly: Sclerotiorine, $C_{20}H_{20}O_5Cl$, A Chlorine-Containing Metabolic oduct of *Penicillium sclerotiorum* Van Beyma, *Biochem. Jour.*, **34**: 1419–21 (1940).

A. W., and R. E. Neidig: The Soluble Polysaccharides of Lower Fungi. Mycodextrin, a New Polysaccharide in *Penicillium expansion, Jour. Biol. em.*, **18**: 167 (1914).

G., J. J. Gallagher, G. T. Newbold, and F. S. Spring: Aspergillic Acid. rt I, *Jour. Chem. Soc.*, 1949, pp. *S*127–*S*131.

—, G. T. Newbold, and F. S. Spring: Aspergillic Acid. Part II. The Conversion of Aspergillic Acid into Leucine and isoLeucine (or *Allo*isoleucine), *r. Chem. Soc.*, 1949, pp. *S*131–*S*133.

y, H. W., E. Chain, N. G. Heatley, M. A. Jennings, A. G. Sanders, E. P.

Abraham, and M. E. Florey: "Antibiotics," Oxford University P York, 1949, Vol. I.

167. Gascoigne, R. M., J. S. E. Holker, B. J. Ralph, and A. Robertson: T istry of Fungi. Part XVI. Eburicoic Acid, *Jour. Chem. Soc.,* 1951, 2352.

168. Gould, B. S., A. A. Tytell, and W. L. Hughes, Jr.: *Abstr. Communs.,* 3 *Congr. Microbiol.,* New York, Sept. 2–9, 1939, p. 51.

169. Haworth, W. N., H. Raistrick, and M. Stacey: Polysaccharides Synth Micro-organisms. I. The Molecular Structure of Mannocarolose from Glucose by *Penicillium charlesii* G. Smith, *Biochem. Jour.,* (1935).

170. ———, ———, and ———: Polysaccharides Synthesized by Micro-o II. The Molecular Structure of Varianose Produced from Glucose by *lium varians* G. Smith, *Biochem. Jour.,* **29**: 2668 (1935).

171. Hind, H. G.: The Colouring Matters of *Penicillium carmino-violaceum* with a Note on the Production of Ergosterol by This Mold, *Bioche* **34**: 67–72 (1940).

172. ———: The Constitution of Carviolin: A Colouring Matter of *Pe* *carmino-violaceum* Biourge, *Biochem. Jour.,* **34**: 577–579 (1940).

173. Igarasi, H.: *Jour. Chem. Soc. Japan,* **15**: 229 (1939).

174. Iwanoff, N. N., and E. S. Zwetkoff: The Biochemistry of the Fungi, *An* *Biochem.,* **5**: 585 (1936).

175. Kinoshita, K.: Über die produktion von Itaconsäure und Mannit durc neuen Schimmelpilze, *Aspergillus itaconicus, Acta Phytochim. Japan,* (1932).

176. Kögl, F., und W. B. Deijs: Untersuchungen über Pilzfarbstoffe. XI Boletol, den Farbstoff der blau anlaufender Boleten, *Ann.,* **515**: 10 (193

177. Linossier, G.: Sur une hématine végétale, l'aspergilline, *Compt. rend.,* 11 (1891).

178. Lockwood, L. B., and A. J. Moyer: The Production of Chemicals by Filam Fungi, *Botan. Rev.,* **4**: 140 (1938).

179. May, O. E., and G. E. Ward: Hydrolysis of the Chitinous Complex of Fungi, *Jour. Am. Chem. Soc.,* **56**: 1597 (1934).

180. Mull, R. P., and F. F. Nord: Structure and Action of Rubrofusari *Fusarium graminearum* Schwabe (Fgra.) (*Gibberella saubinetti*), *Arc chem.,* **4**: 419–433 (1944).

181. Newbold, G. T., W. Sharp, and F. S. Spring: Aspergillic Acid. Part II Synthesis of Racemic Deoxyaspergillic Acid, *Jour. Chem. Soc.,* 1951, pp 2682.

182. Nord, F. F.: Enzymatische Umsetzungen durch Fusarien: Beitrag zum M nismus der alkoholischen Gärung, *Ergeb. Enzymforsch.,* **8**: 149–184 (1

183. ———: On the Mechanism of Alcoholic Fermentation, *Abstr. Com* *3d Intern. Congr. Microbiol.,* New York, Sept. 2–9, 1939, p. 337.

184. Norman, A. G., and W. H. Peterson: The Chemistry of Mold Tissue. I Resistant Cell Wall Material, *Biochem. Jour.,* **26**: 1946 (1932).

185. Posternak, T.: Recherches sur la biochimie des champignons inférieur I la constitution et la synthèse de la phoenicine et sur quelques no dérivés de la 4,4′-ditoluquinone, *Helv. Chim. Acta,* **21**: 1326–1337 (1938).

186. Pritham, G. H., and A. H. Anderson: The Carbon Metabolism of *Fu* *lycopersici* on Glucose, *Jour. Agr. Research,* **55**: 937 (1937).

187. Raistrick, H.: Certain Aspects of the Biochemistry of the Lower ("Moulds"), *Ergeb. Enzymforsch.,* **7**: 316–349 (1938).

188. ———: Biochemistry of the Lower Fungi, *Ann. Rev. Biochem.,* 9: 5 (1940).

189. ———: The Chemistry of Fungi. A Region of Biosynthesis, *Suomen Ke* *ehti,* **10A**: 221–246 (1950).

190. Stodola, F. H., J. L. Wachtel, A. J. Moyer, and R. D. Coghill: Penitrini a New Pigment from *Penicillium notatum, Jour. Biol. Chem.,* **159**(1): (1945).

umiki, Y.: Studies on the Fermentation Products of Mould Fungi. X. Glaucic
Acid Formed by *Aspergillus glaucus* (*Aspergillus glaucus* IV), *Bull. Agr. Chem.
Soc. Japan*, **9**: 105 (1933).

atum, E. L.: Biochemistry of Fungi, *Ann. Rev. Biochem.*, **13**: 667–704 (1944).

hom, C., and K. Raper: The Arsenic Fungi of Gosio, *Science*, **75**: 548 (1932).

——, A. A. Tytell, and W. L. Hughes, Jr.: Studies in the Biochemistry of Fusaria, *Abstr. Communs., 3d Intern. Congr. Microbiol.*, New York, 1939, pp. 51–52.

enck, P. R., W. H. Peterson, and E. B. Fred: The Chemistry of Mold Tissue.
IX. Cultural Factors Influencing the Growth and Sterol Production by *Aspergillus fischeri*, *Zentr. Bakt. Parasitenk.*, Abt. II, **92**: 330 (1935).

——, ——, and H. C. Greene: The Chemistry of Mold Tissue. VIII. Innate
Factors Influencing Growth and Sterol Production of *Aspergillus fischeri*,
*Zentr. Bakt. Parasitenk.*, Abt. II, **92**: 324 (1935).

hite, M. G., and J. J. Willaman: *Fusarium lini* and the Pyruvic Acid Theory
of Alcoholic Fermentation, *Biochem. Jour.*, **22**: 592 (1928).

oolley, D. W., and W. H. Peterson: The Chemistry of Mold Tissue. XI. Isolation of Leucine and Isoleucine from *Aspergillus sydowi*, *Jour. Biol. Chem.*,
**114**: 85 (1936).

unschendorff, M., et C. Killian: Observations sur le métabolisme de l'*Ustulina
vulgaris* L., *Compt. rend.*, **187**: 572 (1928).

## ome References concerning β-Carotene Production by Microorganisms

derson, R. F., M. Arnold, G. E. N. Nelson, and A. Ciegler: Microbial Produc-
ion of Carotenoids. II. The Effects of Selected Medium Adjuncts on β-Caro-
ene Synthesis, *Abstr. 132d Meeting Am. Chem. Soc.*, 1947, p. 17A.

rnett, H. L., V. G. Lilly, and R. F. Krause: Increased Production of Carotene
y Mixed + and − Cultures of *Choanephora cucurbitarum*, *Science*, **123**: 141
1956).

gler, A., M. Arnold, and R. F. Anderson: Microbiological Production of
arotenoids. IV. Effect of Various Grains on the Production of β-Carotene by
icroorganisms, *Bact. Proc.*, 1958*a*, p. 13.

—, ——, and ——: V. Influence of Oils and Fatty Acids on the Produc-
on of β-Carotene by Microorganisms, *Bact. Proc.*, 1958*b*, pp. 13–14.

fel, R. D., and F. M. Clark: Production of β-Carotene by Species in the
enus *Rhodotorula*, *Bact. Proc.*, 1958, p. 13.

seltine, C. W., and R. F. Anderson: Microbiological Production of Carot-
oids. I. Zygospores and Carotene Produced by Intraspecific and Interspe-
fic Crosses of Choanephoraceae in Liquid Media, *Mycologia*, **49**: 449–452
957).

— and C. R. Benjamin: Notes on the Choanephoraceae, *Mycologia*, **49**:
3–733 (1957).

pfer, W. H., et A. Jung: Recherches sur l'activité vitaminique A du thalle
ne Mucorinée, *Compt. rend. soc. biol.*, **120**: 1093 (1935).

## CHAPTER 43

# THE PRODUCTION OF AMINO ACIDS BY MICROORGANISMS

A considerable amount of interest has been displayed in vari
of the world relating to the production of amino acids by ferr
processes, particularly lysine, glutamic acid, and tryptophan.
is being manufactured in the United States by a patented proc
is described below. Plants to produce large amounts of L-gluta
have been constructed in Japan and in the United States. Sweet
are used as the raw material in a fermentation brought about by
requiring strain of *Micrococcus* in the Japanese plant.

## LYSINE

Free extracellular lysine may be produced in very small quanti
comparatively large number of microorganisms. Richard and
(1957) screened over 600 fungi in order to determine their a
form free, extracellular lysine under submerged-culture conditio
a medium containing commercial glucose, urea, and mineral salts
three of the isolates produced sufficient lysine to be detected by a
metric bio-assay method. Twelve fungi produced 5 to 15 γ of ly
milliliter of culture filtrate.

Considerably larger yields of the acid may be obtained by
the best producers and culturing them under optimum conditions.
(1957) obtained yields of about 400 μg. per ml. of extracellular
shaken flasks, when using a strain of a *Gliocladium* species and
of *Ustilago maydis*.

## COMMERCIAL PRODUCTION OF L-LYSINE

A two-stage process is used for the commercial production o
Diaminopimelic acid is first produced. It is then converted to
Methods for this process have been described by Casida (1956).

**Diaminopimelic Acid.** This acid may be produced by a nu
microorganisms. Work (1951) reported on its production by
*bacterium diphtheriae* and *Mycobacterium tuberculosum*. Davis
noted that relatively large amounts of the acid were produ
some lysine-requiring auxotrophs of *Escherichia coli*. Casida (195

train of *E. coli* (now designated as A.T.C.C. 12,408) which required
ne and which was unable to decarboxylate diaminopimelic acid to
ne.

)iaminopimelic acid is produced by submerged-culture methods. Ac-
ling to Casida, the use of 1 to 10 per cent by volume of glycerol and
ı-steep liquor (as a source of lysine) and the control of the pH are
: important considerations in its preparation.

he inoculum for the production medium may be prepared by growing
*oli* A.T.C.C. 12,408 for 20 hr. at 28°C. with agitation and aeration in
edium of the following composition:

| | Per Cent |
|---|---|
| Glycerol | 0.5 |
| Corn-steep liquor | 0.5 |
| $(NH_4)_2HPO_4$ | 0.5 |

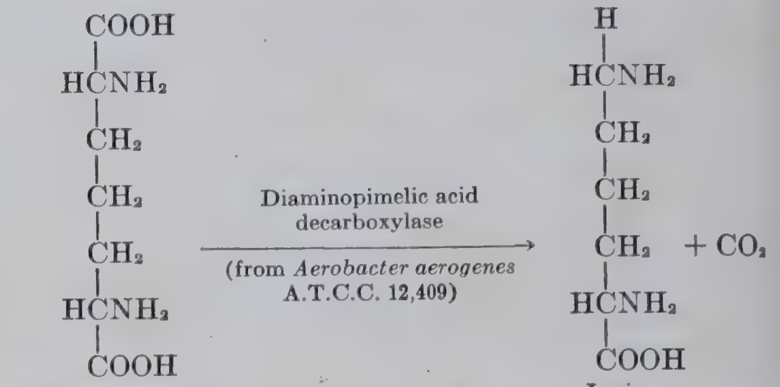
pH of the medium is adjusted to 7.5 with KOH. The medium is ster-
l at a pressure of 20 lb. per sq. in. for 30 min.

ıe production medium, cited as an example in the patent issued to
la, contained 4 per cent of $(NH_4)_2HPO_4$, 4 per cent by volume of
-steep liquor, 6 per cent of glycerol, and 0.5 per cent of $CaCO_3$. The
f the medium was adjusted to 7.5 with KOH, and the medium was
ized. The medium may be seeded with 1 to 5 per cent of inoculum
ncubated at 28°C. with agitation and aeration (1 volume per vol-
of medium per minute). Traces of soybean oil or DC Antifoam A
be added as an antifoam agent.

ring the fermentation, it is important to maintain the pH in the
of 7 to 7.5. After about 24 hr., it is necessary to add an alkali,
nium hydroxide being preferred (but KOH and NaOH are satis-
y); after about 64 hr., it may be necessary to add $H_2SO_4$.
imum yields (about 9 mg. per ml.) are obtained in about 3 days.

**minopimelic Acid Decarboxylase.** This enzyme, which converts
ıopimelic acid to lysine, is produced by a number of bacteria (Work,
for example, by strains of *Aerobacter aerogenes* and of *E. coli*
do not require lysine for growth. Casida reported that a strain of
*ogenes* (now designated as A.T.C.C. 12,409) was particularly useful
ıverting diaminopimelic acid to lysine. This organism lacked the
e lysine decarboxylase.

*erogenes* A.T.C.C. 12,409 may be grown in the same medium and
similar conditions to those found favorable for the preparation of
culum for the mutant strain of *E. coli*. After propagation, the cells
parated from the medium by centrifuging and used directly or
dried and used as required. In one example quoted by Casida, the
om 25 ml. of growth medium were added to a 300-ml. Erlenmeyer
vhich contained 100 ml. of broth with diaminopimelic acid at a
7.2 and 5 ml. of toluene (which liberates the enzyme). The flask
contents was shaken for about 16 hr. at 28°C. for the conversion,
roceeds in accordance with the following scheme:



Diaminopimelic acid

Lysine

In general, the conversion of diaminopimelic acid to lysi
carried out at about 28°C. in approximately 24 hr. The a
0.004 to about 0.032 molar citric acid and ethylenediamine
acid tetrasodium salt favors the conversion, particularly whe
centration of diaminopimelic acid is high. The addition of
vitamin $B_6$ also aids the reaction, according to Casida. The en
be liberated from the bacterial cells by treatment with butano
sonic vibrations or as a result of the preparation of the ace
powder, in addition to the use of toluene described herein.

After conversion, the reaction mixture is filtered, and the
absorbed on a strong cation-exchange resin, such as Amberlit
eluted with dilute alkali, passed through a weak cation-excha
such as Amberlite IRC-50, and dried. Further purification may

## L-GLUTAMIC ACID

L-Glutamic acid may be produced in a number of ways: by the
sis of wheat gluten, soybean cake, or other protein-rich food ma
cleavage of the pyrrolidone carboxylic acid found in Steffen's
by a one-stage fermentation process using a single microorgani
two-stage process involving the production of α-ketoglutaric
fermentation and its conversion by a second microbe or by an
process; and by enzymes.

## ONE-STAGE FERMENTATION PROCESSES

**Commercial Process.** The commercial process now being
Japan employs sweet potatoes as the chief raw material and is
Commonwealth of Australia patent applications (1956*a*, *b*, *c*).

Study of the patents reveals that a strain of *Micrococcus*,
which requires 0.5 to 5 γ per liter of biotin for good yields, is u
carbon source may contain glucose, fructose, sucrose, maltos
or hydrolyzed starch. The nitrogen source should contain at lea
the following substances: urea, ammonia, ammonium salts, pept
steep liquor, hydrolyzed casein, meat extract, or digested soyb
or fish meal. The pH should be kept in the range of 6 to 9, but
mum range is 7 to 8.5. The desired pH may be maintained th

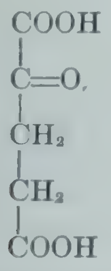
of ammonia, urea, a compound containing basic nitrogenous radical, caustic alkalis. The ammonium ion is essential. The temperature is intained at 27 to 30°C. Aerobic conditions are used. Yields of 21 to per cent, based on the glucose consumed, are reported in the patent lications. α-Ketoglutaric acid is produced by the *Micrococcus* and is eved to be the precursor of the L-glutamic acid.

**aboratory Method.** Asai, Aida, and Oishi (1957) described a labory method for producing L-glutamic acid. Microbes belonging to a iber of different genera of bacteria and molds were found to be able ccumulate L-glutamic acid. However, strains of the genera *Micro- us* and *Bacillus* appeared to be best. *M. varians* was used in experits in which the following medium was employed: glucose, 20 g.; ionium chloride, 7 g.; $KH_2PO_4$, 1 g.; and $MgSO_4 \cdot 7H_2O$, 0.05 g. per . The pH was adjusted to 7, and 0.5 per cent of separately sterilized $O_3$ was added before inoculation. Fermentations were carried out 200-ml. portions of the medium contained in 500-ml. Erlenmeyer s, incubated at 30°C. on a rotary shaker. A yield of 23 per cent itamic acid, based on the glucose supplied, was obtained in one riment. α-Ketoglutaric acid was detected in small amounts.

**oduction by Cephalosporium Species.** A method for the direct iction of L-glutamic acid by means of a strain of *Cephalosporium,* as *C. salmosynnematum* NRRL 2271, *C. diospyri* A.T.C.C. 9066, or *remorium* A.T.C.C. 10141, has been patented by Kita (1957). The is cultivated in a nutrient medium containing a carbohydrate source irea at a pH in the range of 4 to 9 under aerobic conditions until ciable amounts of L-glutamic acid accumulate.

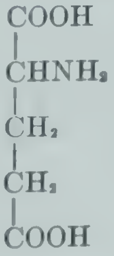
## TWO-STAGE PROCESSES

microbiological production of L-glutamic acid from α-ketoglutaric ivolves two steps: one in which α-ketoglutaric acid is formed under im conditions by the use of a selected microorganism and one in the α-ketoglutaric acid, thus formed, is converted to L-glutamic y a second microbe or by enzymic means.



$$\begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{C{=}O}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{COOH}\end{array}$$

α-Ketoglutaric acid



$$\begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{CHNH_2}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{COOH}\end{array}$$

L-Glutamic acid

**uction of α-Ketoglutaric Acid.** The production of α-ketoglutaric fermentation has been described by Lockwood and Stodola (1946, Koepsell, Stodola, and Sharpe (1952); Asai and Aida (1952); ida, and Sugisaki (1953, 1954); Sugisaki, Aida, and Asai (1955); igisaki, and Aida (1955); Masuo and Wakisaka (1955); Asai,

TABLE 202. DATA CONCERNING THE PRODUCTION OF α-KETOGLUTARIC ACID BY FERMENTATION

| Microorganism | | Approx. maximum yield reported | Reference |
|---|---|---|---|
| Strain no. | Name | | |
| | *Bacterium α-ketoglutaricum* | 50–60% | Masuo, E., and Y. Wakisaka: *Jour. Agr. Chem. Soc. Japan,* **29**: 476, 550 (1955). |
| | *Bact. succinicum* * | 13.6% | Sakaguchi and Tada: *Zentr. Bakt. Parasitenk.,* Abt. II, **101**: 341 (1940). |
| 1 | *Bacillus megatherium* * | 20.6% | Asai, T., K. Aida, and Z. Sugisaki: *Jour. Agr. Chem. Soc. Japan,* **27**: 129 (1953). |
| | *B. natto* (var. *B. subtilis*) | 14.6% | |
| | Bacteria of *coli-aerogenes* group (*Escherichia, Aerobacter, Proteus*) | 2,384 mg. in 100-ml. fermentation liquid | Katagiri, H., K. Imai, and T. Tochikura: U.S. Patent 2,786,799, Mar. 26, 1957. |
| 3 | *Escherichia coli* | 40–60% | Asai, T., S. Okumura, and T. Tsunoda: *Jour. Gen. Appl. Microbiol.,* **3**(1): 13 (1957). |
| 7 | *E. coli* | 40–60% | |
| 5 | *E. freundii* | 40–60% | |
| 9 | *E. freundii* | 40–60% | |
| 12 | *E. freundii* | 40–60% | |
| 8 | *Aerobacter cloacae* | 40–60% | |
| 84C | *Kluyvera citrophila* var. alpha | 40–60% | |
| 11 | *K. citrophila* var. α | 40–60% | |
| 6 | *K. citrophila* var. beta | 40–60% | |
| 4 | *K. noncitrophila* | 40–60% | |
| 10 | *K. noncitrophila* | 40–60% | |

|  |  |  |  |
|---|---|---|---|
|  | *Gluconobacter cerinum* * | About 36% | Asai, T., Z. Sugisaki, and K. Aida: *Jour. Agr. Chem. Soc. Japan,* **29**: 300 (1955). |
|  | *Pseudomonas fluorescens* | 16.8% based on glucose available | Lockwood, L. B., and F. H. Stodola: U.S. Patent 2,443,919, June 22, 1948. |
| 33F | *Ps. fluorescens* | 16.4% | Asai, T., Z. Sugisaki, and N. Yakeishi: *Jour. Gen. Appl. Microbiol.,* **1**(4): 308 (1955). |
| NRRL B-6 | *Ps. fluorescens (reptilivora)* | 47.9% | Koepsell, H. J., et al.: *Jour. Am. Chem. Soc.,* **74**: 5142 (1952). |
| 8UK<br>NRRL B-1481 | *Serratia marcescens* | 33.3% based on initial glucose | Sharpe, E. S., and J. Corman: U.S. Patent 2,776,926, Jan. 8, 1957. |
| 18 | *S. marcescens* | 49.3% | Asai, T., et al.: *Jour. Gen. Appl. Microbiol.,* **1**(4): 308 (1955). |

* Refer also to T. Asai et al., *Jour. Gen. Appl. Microbiol.,* **1**(4): 308 (1955).

Aida, Sugisaki, and Yakeishi (1955); Koepsell and Stodol
Sharpe and Corman (1957); Asai, Okumura, and Tsunoda (19
giri, Imai, and Tochikura (1957); Otsuka, Yazaki, Nagase, and
(1957*a*, *b*, *c*, *d*); and others.

*Microorganisms Producing.* Although considerable screenin
has been accomplished regarding α-ketoglutaric acid prod
microbes, there remain much to be done and many possibiliti
ence to Table 202 will indicate the kinds of microorganisms w
been studied and which show promise in regard to this fermenta

*Nutrients.* The usual production medium contains a carbon
nitrogen source, minerals, and, occasionally, vitamins.

Glucose is one of the most widely used carbon sources. Howe
sugars, such as fructose and mannose, and, occasionally, orga
such as acetic and succinic, may serve as satisfactory carbon so

Nitrogen sources may include one or more of the following ing
ammonium sulfate, ammonium nitrate, ammonium phosphate,
urea, amino acids, casein, soybean cake, fish meal, or other com

When synthetic or semisynthetic media are used, magnesium
potassium acid phosphate, and various other minerals may be
to the medium. A trace of iron may enhance yields; for exan
addition of 8 p.p.m. of ferric sulfate to a nutrient medium
increased the amount of α-ketoglutaric acid produced by *Serratia*
*cens* (Asai et al., 1955).

Yeast extract is used in some media as a source of vitamins a
nutrients.

Calcium carbonate is often used as a neutralizing agent and b

Examples of media used in the production of α-ketoglutaric
given in Table 203.

*Production by* Pseudomonas fluorescens. Koepsell, Stodola, and
(1952) studied the production of α-ketoglutaric acid, using *Ps. flu*
B-6. The medium used by them is shown in Table 203. It was d
in 200-ml. portions in 1-liter Erlenmeyer flasks, sterilized, cooled
lated, and incubated at 28°C. on a reciprocating shaker. Data
by Koepsell et al. are presented in Table 204.

The amount of nitrogen supplied showed a marked effect on t
of α-ketoglutarate. When the level of nitrogen was less than 25
ml. (calculated as 1 g.-atom per mole), incomplete fermentation r
when it was above 50 μM. per ml., yields of α-ketoglutarate w
or absent, but fermentation was considerably more rapid. A conce
of 25 μM. per ml. was used in most of the studies.

As a result of their studies, Koepsell and associates report
α-ketoglutarate may be produced in relatively high yield, inexpe
and with comparative ease on media of simple composition.

*Production by* Kluyvera citrophila *var. α.* A considerable am
significant research on the α-ketoglutaric acid fermentation, part
relating to the effects of various nutrients on yields, has been m
Asai and associates (1955).

They obtained yields of up to 56 per cent of α-ketoglutaric ac

| Medium | Amount | Microbe | Temp., °C. | Time of fermentation, hr.* | Max. yield of α-ketoglutaric acid, % | Reference |
|---|---|---|---|---|---|---|
| Glucose ....... | 4.57% | *Serratia marcescens* | 30 | 144 | 49.3 | Asai, T., et al.: *Jour. Gen. Appl. Microbiol.*, **1**(4): 306–346 (1955). |
| Urea ......... | 0.07% | | | | | |
| $KH_2PO_4$ ...... | 0.05% | | | | | |
| $MgSO_4 \cdot 7H_2O$ .. | 0.01% | | | | | |
| $CaCO_3$ † ...... | 1.25% | | | | | |
| $Fe_2(SO_4)_3$ ..... | 8 p.p.m. | | | | | |
| Fructose ...... | 4.93% | *Kluyvera citrophila* var. α (*Bacterium* No. 84C) | 30 | 89 | 57.0 | Asai, T., et al.: *Jour. Gen. Appl. Microbiol.*, **1**(4): 308–346 (1955). |
| $NH_4H_2PO_4$ .... | 0.12% | | | | | |
| $(NH_4)_2SO_4$ .... | 0.12% | | | | | |
| $KH_2PO_4$ ...... | 0.10% | | | | | |
| $MgSO_4 \cdot 7H_2O$ .. | 0.04% | | | | | |
| NaCl ......... | 0.30% | | | | | |
| $CaCO_3$ † ...... | 4% | | | | | |
| $FeSO_4 \cdot 7H_2O$ ... | 1 p.p.m. | | | | | |
| Glucose ....... | 9 g./100 ml. | *Pseudomonas fluorescens* B-6 | 28 | 263 | 47.9 | Koepsell, **H. J., F. H.** Stodola, and E. S. Sharpe: *Jour. Am. Chem. Soc.*, **74**: 5142–5143 (1952). |
| $(NH_4)_2SO_4$ ‡ | 0.16–0.32 g./100 ml. | | | | | |
| $KH_2PO_4$ ...... | 0.11 g./100 ml. | | | | | |
| $MgSO_4$ ....... | 0.024 g./100 ml | | | | | |
| $CaCO_3$ † ...... | 3.75 g./100 ml. | | | | | |
| Fe § .......... | 1 p.p.m. | | | | | |

* In shaken flasks.

† Sterilized separately.

‡ Or equimolar amounts of urea.

§ Supplied as ferrous ammonium sulfate.

Table 204. Yields of α-Ketoglutarate and Pyruvate in Glucose Ox
*Pseudomonas fluorescens* *

| Experiment no. | Fermentation time, hr. | Initial glucose, μM./ml. | Final 2-ketogluconate, μM./ml. | Pyruvate found | | α-Keto fo |
|---|---|---|---|---|---|---|
| | | | | μM./ml. | Molar yield | μM./ml. |
| 151 | 307 | 510 | 0 | 3 | 0.01 | 281 |
| 152 | 386 | 513 | 0 | 5 | 0.01 | 252 |
| 154 | 310 | 506 | 8 | 5 | 0.01 | 260 |
| 154 | 310 | 510 | 9 | 6 | 0.01 | 259 |
| 157 | 384 | 510 | 0 | 0 | 0.00 | 241 |
| 152 | 288 | 512 | 28 | 52 | 0.11 | 227 |
| 146 | 263 | 510 | 102 | 62 | 0.15 | 242 |
| 159 | 216 | 510 | 100 | 13 | 0.03 | 223 |

* H. J. Koepsell, F. H. Stodola, and E. S. Sharpe, *Jour. Am. Chem. Soc.*, (1952). Copyright 1952 by the American Chemical Society and reprinted b sion of the copyright owner.

† A yield of 0.5 mole of α-ketoglutaric acid per mole glucose corresponds t of 40.6 g. per 100 g. of glucose.

a 10.4 per cent glucose solution after 72 hr. with *Bacterium* strain (*K. citrophila* var. α), which was isolated from the soil and descr them. The medium used for securing maximum yields contained glucose) 0.135 g. $(NH_4)_2SO_4$, 0.135 g. $(NH_4)H_2PO_4$, 0.04 g. $MgSO_4$ 0.1 g. $KH_2PO_4$, and 2 p.p.m. $FeSO_4 \cdot 7H_2O$ in 100 ml. of distille (before incubation, 8 g. of sterile $CaCO_3$ was added). Fifty-ml. of the medium were dispensed in 500-ml. flasks, sterilized, inoculat incubated at 30°C. on a reciprocating shaker moving at 116 r.p.m.

Glucose, fructose, and the acid hydrolyzate of starch were obse be excellent carbon sources, whereas galactose, sucrose, and malto poor sources.

A nitrogen concentration of about 0.05 per cent was found to mum for the production of the highest yields of α-ketoglutaric this organism. Ammonium salts were effective nitrogen sources, nium sulfate, ammonium nitrate, and ammonium dihydrogen ph being especially good. Urea was also satisfactory. Corn-steep liq polypeptone were unsatisfactory as nitrogen sources.

**Conversion of α-Ketoglutaric Acid to L-Glutamic Acid.** Th version may be brought about by microorganisms or by enzymic

*Conversion by Microorganisms.* Otsuka and associates (1957 have carried out extensive research concerning the use of micr effect the conversion of α-ketoglutaric acid and an ammonium L-glutamic acid. They carried out a screening program to find org capable of making the conversion and studied the best one of these

MEDIA. They made use of three media, designated as $A_1$, $A_2$, The composition of the media follows:

ium $A_1$:
dium-α-ketoglutarate, 1% (as free acid)
ucose, 0.1%
$_4$Cl, 0.5%
$HPO_4$, 0.1%
$SO_4 \cdot 7H_2O$, 0.05%
um $A_2$:
dium $A_1$ plus 0.01% casein-tryptic-hydrolyzate
um $A_3$:
dium $A_1$ plus 0.008% casein-tryptic-hydrolyzate plus 0.007% yeast extract

1 mixture of distilled water and tap water was used in making up the
a. The pH of each medium was adjusted to 7.

EENING. Microbes were isolated from the soil by use of the fore-
media solidified with agar. Plates were incubated at 25, 30, and
. for 48 hr. Tubes of liquid media were inoculated from the isolated
res and incubated at the same temperatures for 5 days. The cells
then separated by centrifuging, and the supernatants were examined
per chromatography for glutamic acid production.

uka et al. examined 1,599 isolates. Only 62 strains produced glu-
acid in a concentration greater than 5 μM. per ml. of culture
m.

ERA PRODUCING. Cultures belonging to the following genera were
o accumulate glutamic acid: *Pseudomonas, Xanthomonas, Erwinia,*
*ia, Bacillus, Micrococcus, Escherichia, Aerobacter, Hansenula,*
*yomyces, Mycotorula, Saccharomyces,* and other yeasts and *Asper-*
*Penicillium,* and *Rhizopus. Ps. ovalis* No. 259-2 showed the high-
el of glutamic acid accumulation.

MUM CONDITIONS FOR CONVERSION BY *Ps. ovalis.* Otsuka and asso-
(1957*a*) tested a number of nitrogen sources, including ammonium
ate, ammonium sulfate, ammonium acetate, ammonium nitrate,
ium citrate, ammonium chloride, and urea and found that ammo-
hloride was best.

er studies revealed that aerobic conditions favored the growth of
*lis,* but not glutamic acid accumulation. Consequently the organ-
s grown in medium $A_3$ for 17 to 18 hr. at 30°C. on a shaker and
parated from the medium by centrifuging. The cells were washed
mes, added to a new batch of medium $A_3$, and incubated at 30°C.
ically.

est yields of glutamic acid were obtained when (1) incubation was
lished under anaerobic conditions at 30°C. and (2) the pH of the
was maintained at neutrality or slightly on the alkaline side
s. Usually about 98 per cent of the α-ketoglutarate added was
d within 24 to 30 hr., and 30 μM. per ml. of glutamic acid accu-
in the medium. This corresponded to about 60 per cent of the
d α-ketoglutaric acid. On the basis of yields of 50 to 60 per cent
oglutaric acid from glucose, this would be equivalent to an over-
of about 30 per cent from glucose.

*rsion by Enzymes.* Enzymatic methods for converting α-keto-
acid to glutamic acid have been developed.

Smythe and Huang (1956*a*) patented a process for converti glutaric acid and ammonium ions to glutamic acid, using (1) a catalytic system, containing a reversibly oxidizable and redu tinic-acid-containing coenzyme, (2) a water-soluble hydroge reactant, and (3) a dehydrogenase system specific for this rea hydrogen donor reduces the oxidized coenzyme of the dehydrog glutamic acid is formed. The reaction is carried out at a pH o and in the temperature range of 20 to 45°C.

The biological catalytic system is selected from at least one a group consisting of extracts from heart, liver, and kidney; l the genera *Xanthomonas* and *Pseudomonas;* lysed fungi of *niger, A. aureus,* and *A. wentii,* of *Neurospora crassa,* of *Botry* and of *Penicillium glaucum;* and lysed yeasts of *Torula cr Candida* (*Torulopsis*) *utilis,* of *Saccharomyces fragilis,* and of *S.*

The hydrogen-donating reactant may be citric acid, mali glucose-6-phosphate.

## ENZYMATIC PROCESSES

**Preparation from Citrate and Ammonium Ions.** Methods fo ing L-glutamic acid from citrate and ammonium ions by the use o system, with or without the addition of a coenzyme system, h described by Smythe and Huang (1956*b*) and Huang and Smyth

Special conditions are used during production. The amount (trisodium citrate, for example) used is in the range of 0.01 to 0 but that preferred is 0.05 to 0.15 mole. At least 1 mole of ammon (ammonium chloride, for example) is used for each mole of cit A concentration of iron (supplied as ferrous ammonium su 1 p.p.m. is desirable. The source (or sources) of the enzyme a zyme systems is added to the aqueous solution, which is maintain pH range of 6 to 8.5 (7 to 8 preferred) and at a temperature in of 20 to 40°C. (25 to 35°C. preferred).

In one method (Smythe and Huang, 1956*b*), lysed or autoly (*Saccharomyces cerevisiae, S. fragilis, Torula cremoris,* or *Candi* is used as the source of the enzyme system.

In another method (Huang and Smythe, 1957), the enzyme supplied through the use of viable, autolyzed, or lysed cells of *Pseudomonas* or *Xanthomonas,* for example, *Ps. fluorescens* NRR The coenzyme is derived from liver or yeast.

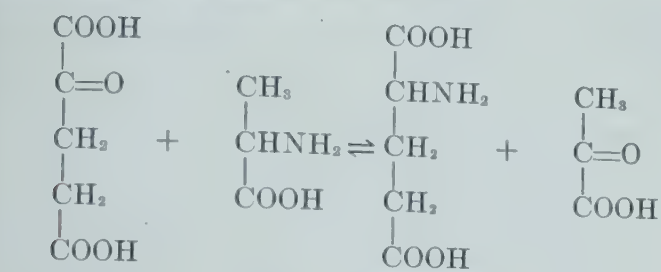
High yields are reported for these methods, amounting to 80 t cent in some cases, based on the available citrate.

## SOME POSSIBLE PATHWAYS OF FORMATION

It is generally accepted that α-ketoglutaric acid is the pre L-glutamic acid. The α-ketoglutaric acid is believed to arise fro by way of the tricarboxylic acid cycle through pyruvate an (refer to page 569).

-Glutamic acid may arise from α-ketoglutaric acid by two or more ways. In one pathway, α-ketoglutaric acid is acted upon by L-glu- ic dehydrogenase in the presence of reduced triphosphopyridine eotide ($TPNH_2$) and ammonia and gives rise to L-glutamic acid, N, and water. In addition to the glutamic acid dehydrogenase, iso- te dehydrogenase may also be involved (Kinoshita et al., 1957).

a second pathway, glutamic acid may arise by a transamination tion. In this type of reaction, an amino acid acts as a donor of an o group to a second compound, known as the "acceptor." The reac- is catalyzed by the enzyme transaminase, with the aid of pyridoxal phate, a coenzyme. An example follows:


α-Ketoglutaric acid    Alanine    L-Glutamic acid    Pyruvic acid

ing their opinion on experimental studies, Kinoshita and associates at the first pathway was the important one.

litional information on this subject may be obtained by reference to blications of Roberts et al. (1955), Thimann (1955), Clifton (1957), hita et al. (1957), and others.

## eferences concerned with the Microbiological Production of Amino Acids

. and K. Aida: On the Production of α-Ketoglutaric and Pyruvic Acids by *domonas* and *Serratia*, *Jour. Agr. Chem. Soc. Japan*, **26**: 528 (1952). (In anese.)

——, and K. Oishi: On L-Glutamic Acid Fermentation, *Bull. Agr. Chem Japan*, **21**(2): 134–135 (1957).

——, and Z. Sugisaki: On the Production of α-Ketoglutaric Acid by *llus megaterium* and *Bacillus natto*, *Jour. Agr. Chem. Soc. Japan*, **27**: 129 3). (In Japanese.)

——, and ——: On the α-Ketoglutaric Acid Fermentation, *Jour. Agr. n. Soc. Japan*, **28**: 479 (1954); **29**: 296 (1955). (In Japanese.)

——, ——, and N. Yakeishi: On α-Ketoglutaric Acid Fermentation, *Gen. Appl. Microbiol.*, **1**(4): 308–346 (1955).

S. Okumura, and T. Tsunoda: On the Classification of the α-Ketoglutaric Accumulating Bacteria in Aerobic Fermentation, *Jour. Gen. Appl. Micro-* , **3**(1): 13–33 (1957).

. Sugisaki, and K. Aida: The Formation of α-Ketoglutaric Acid and Pyruvic by *Gluconoacetobacter cerinum*, *Jour. Agr. Chem. Soc. Japan*, **29**: 300 ). (In Japanese.)

. E., Jr.: Preparation of Diaminopimelic Acid and Lysine, U.S. Patent 396, Nov. 20, 1956.

. E.: "Introduction to Bacterial Physiology," McGraw-Hill Book Company, New York, 1957.

D.: *Nature* (London): **169**: 534 (1952).

E. L.: Formation of Extracellular Lysine by *Ustilago maydis* and *Glio- m* Sp., *Can. Jour. Microbiol.*, **3**: 467–476 (1957).

Gale, E. F.: Determination of Amino Acids by Use of Bacterial Amino
boxylases, "Methods of Biochemical Analysis," Interscience Publishe
York, 1957, Vol. IV, pp. 285–306.

Huang, H. T., and C. V. Smythe: Production of L-Glutamic Acid,
2,798,839, July 9, 1957.

Katagiri, H., K. Imai, and T. Tochikura: Process for Producing of α-
Acid by Bacteria of the Coli-Aerogenes Group, U.S. Patent 2,786,799, M

Kinoshita, S., K. Nakayama, and S. Akita: Taxonomical Study of Gl
Accumulating Bacteria, *Micrococcus glutamicus* nov. sp., *Bull. Agr.*
*Japan,* 22(3): 176–185 (1958).

———, T. Tanaka, S. Udaka, and S. Akita: 403. Glutamic Acid Fermenta
*Congr. Enzyme Chem., Tokyo,* Oct. 22, 1957.

———, S. Udaka, and M. Shimono: Part I. Production of L-Glutamic Ac
ous Microorganisms, *Jour. Gen. Appl. Microbiol.,* 3(3): 193–205 (195

Kita, D. A.: Production of Glutamic Acid by *Cephalosporium,* U.S. Pate
Apr. 23, 1957.

Koepsell, H. J., and F. H. Stodola: Preparation and Recovery of α-Ketogl
U.S. Patent 2,724,680, Nov. 22, 1955.

———, ———, and E. S. Sharpe: Production of α-Ketoglutaric Acid in G
dation by *Pseudomonas fluorescens, Jour. Am. Chem. Soc.,* 74: 5142–5

Kyowa Kakko Kogyo Kabushiki Kaisha: L-Glutamic Acid and Method of
the Same, Commonwealth of Australia, Patent Applications 23,556
Nov. 28, 1956.

Lockwood, L. B., and F. H. Stodola: Preliminary Studies on the Production
glutaric Acid by *Pseudomonas fluorescens, Jour. Biol. Chem.,* 164: 81-

——— and ———: Fermentation Process for Production of α-Ketoglut
U.S. Patent 2,443,919, June 22, 1948.

Masuo, E., and Y. Wakisaka: Production of α-Ketoglutaric Acid by *Bacteri*
*glutaricum, Jour. Agr. Chem. Soc. Japan,* 29: 476, 550 (1955). (In Japa

Otsuka, S.-I., H. Yazaki, H. Nagase, and K.-I. Sakaguchi: Fermentative
of L-Glutamic Acid from α-Ketoglutaric Acid and Ammonium Salt,
*Appl. Microbiol.,* 3: 35–53 (1957*a*).

———, ———, ———, and ———: Fermentative Production of L-Glut
from α-Ketoglutaric Acid and Ammonium Salt, *Bull. Agr. Chem. S*
21: 69–70 (1957*b*).

———, ———, ———, and ———: Fermentative Production of L-Glutamic
α-Ketoglutaric Acid by Microorganisms, *Jour. Fermentation Assoc. Ja*
54–62 (1957*c*). (Much of article in Japanese.)

———, ———, ———, and ———: L-Glutamic Acid Fermentation, Fe
Production of L-Glutamic Acid from α-Ketoglutaric Acid by Micro
*Jour. Fermentation Assoc. Japan,* 15: 54–62 (1957*d*). (In Japanese.)

Richards, M., and R. H. Haskins: Extracellular Lysine Production by Vari
*Can. Jour. Microbiol.,* 3: 543–546 (1957).

Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britte
of Biosynthesis in *Escherichia coli, Carnegie Inst. Wash. Publ.,* 607, 19

Sharpe, E. S., and J. Corman: Preparation of Alpha-ketoglutaric Acid b
*marcescens,* U.S. Patent 2,776,926, Jan. 8, 1957.

Smythe, C. V., and H. T. Huang: Enzymatic Production of L-Glutamic
Patent 2,749,279, June 5, 1956*a*.

——— and ———: Method of Preparing Citrate Ion-derived L-Glutamic
Patent 2,773,001, Dec. 4, 1956*b*.

Sugisaki, Z., K. Aida, and T. Asai: On α-Ketoglutaric Acid Fermentatio
*Jour. Agr. Chem. Soc. Japan,* 29: 296 (1955). (In Japanese.)

Thimann, K. V.: "The Life of Bacteria," The Macmillan Company, New

Work, E.: Isolation of α,ε-diaminopimelic acid from *Corynebacterium d*
and *Mycobacterium tuberculosis, Biochem. Jour.,* 49: 17–23 (1951).

———: *Biochim. biophys. Acta,* 17: 410 (1955).

# CHAPTER 44

# THE MICROBIOLOGICAL TRANSFORMATION OF STEROIDS

he history of the development of steroids is a fascinating and most ortant one in the fields of medicine, chemistry, and industrial microgy. The use of steroids for the treatment of diseases, some of them erly recalcitrant, has expanded greatly during the past few years. types of steroids with greater therapeutic value and with fewer comting side reactions have been and are being developed. The greatly ased market for steroids has increased the need for larger quantities em at a lower cost.

e steroids used in the field of medicine are generally prepared by a ination of chemical and microbiological methods. Such factors as ase of bringing about a necessary transformation, the cost of the rsion, the time required, and the absence of complicating reactions considered in determining whether the cal or microbiological method will be

robiological conversions of steroids are t about by the enzymes secreted by sestrains of microorganisms. Reviews conwith certain aspects of this subject have nade by Peterson (1953), Finch (1954), . (1954), Hanč and Riedl-Tůmová (1954), y and Hickey (1954), Fried et al. (1955), on (1955), Wettstein (1955), Eppstein (1956), Peterson (1956), and Shull .

structural formula for the basic $\Delta^4$-3-eroid with attached side chain is shown 115.

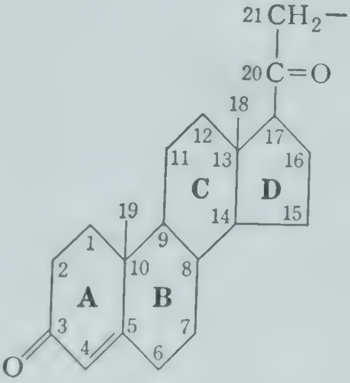


Fig. 115. Basic $\Delta^4$-3-ketosteroid with side chain. The method of numbering the carbon atoms and of lettering the rings is shown.

first successful attempts to transform microbiologically were made by Mamoli and Vercellone (1937), ported that a nuclear hydroxyl group could be oxidized and a double bond could be reduced by yeast. Welsch and Heusghem using *Streptomyces*, confirmed and enlarged upon their results. (1948) demonstrated that cholestenone could be converted to enic, isocaproic, and Windaus acids through the action of a *omyces* species.

Krámli and Horváth (1949) announced that *P. roseus* and
*bacter* species were able to oxidize the nuclear carbon atom in
of the cholesterol molecule to produce 7-hydroxycholesterol.

In 1949, Hench and associates reported that cortisone (and lat
cortisone) produced a remarkable effect on rheumatoid arthritis.
use in the treatment of asthma, allergies, Still's disease, ulcerati
and other diseases was reported.

The presence of the 11-oxygen atom in cortisone and hydro
is essential for the treatment of rheumatoid arthritis and other
The chemical introduction of oxygen at carbon atom 11 is achi
with much difficulty and at large cost.

Peterson and Murray (1952) reported that it was possible to
steroids at nuclear positions other than the 7-position through t
microorganisms. They were able to produce 11α-hydroxyprog
from progesterone with the use of *Rhizopus arrhizus*. Microbiolog
genations of other steroids at the carbon-11 position was acco
using molds of the order Mucorales (class Phycomycetes). Their
were confirmed by Mancera et al. (1952) and by Kahnt and a
(1952). The oxygenation of steroids at the 11-position by a s
*Aspergillus* was reported independently by Fried et al. (1952).
the same year, Colingsworth and coworkers showed that Rei
compound S could be transformed to hydrocortisone by *S. fradiae*
dramatic discoveries were followed by other significant microb
steroid transformations.

11α-Hydroxyprogesterone may be converted to cortisone (
et al., 1952; Peterson, 1953), or to hydrocortisone (Levin et al
readily and economically, according to Peterson (1955).

Different microorganisms are able to act upon the same ste
produce different types of transformations. Figure 116 shows som
steroids produced from progesterone by microbiological transfor
Figure 117 shows some of the steroids which may be formed from
stein's compound S. The possibilities in this field are almost limit

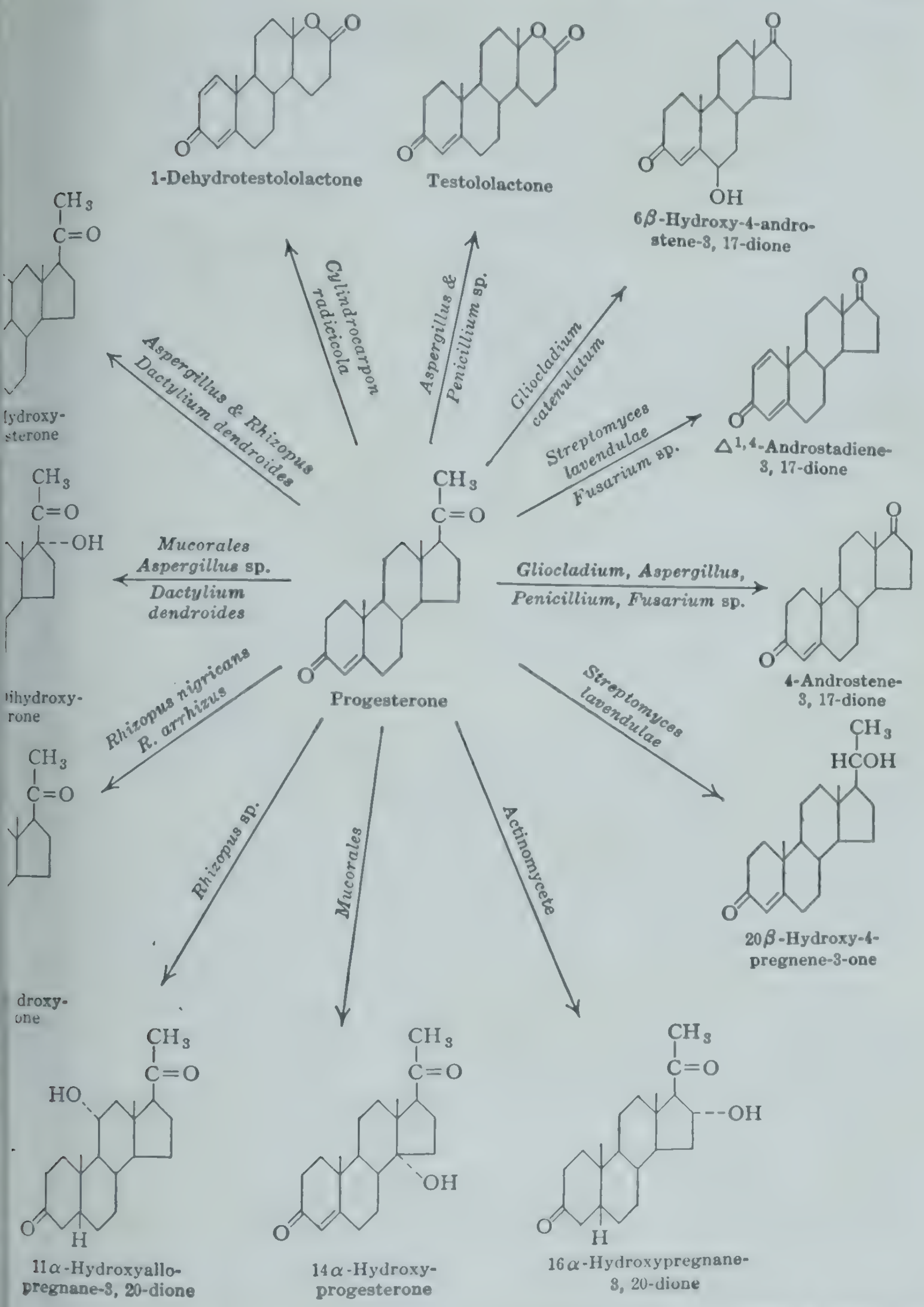
Table 205 gives some synonyms for steroid names.

TABLE 205. SOME SYNONYMS FOR STEROID NAMES

Cortexone = 11-deoxycorticosterone (DOC) = $\Delta^4$-pregnene-21-ol-3,20-dione
Corticosterone = $\Delta^4$-pregnene-11β,21-diol-3,20-dione
Cortisone = Kendall's compound E = $\Delta^4$-pregnene-17β,21-diol,3,11,20-trione
11-Epi-compound F = $\Delta^4$-pregnene-11α,17α,21-triol-3,20-dione
Hydrocortisone = cortisol = 17α-hydroxycorticosterone = Kendall's compou
Prednisolone = $\Delta^{1,4}$-pregnadiene-11β,17α,21-triol-3,20-dione
Prednisone = $\Delta^{1,4}$-pregnadiene-17α,21-diol-3,11,20-trione
Progesterone = $\Delta^4$-pregnene-3,20-dione
Reichstein's compound S = 17α-hydroxy-11-deoxycorticosterone = deoxyc
17α-hydroxy-cortexone = $\Delta^4$-pregnene-17α,21-diol-3,20-dione

## METHODS OF TRANSFORMATIONS

**General.** The microorganism of choice is grown in a specially
medium which will support good growth, such as one of those


1-Dehydrotestololactone
Testololactone
6β-Hydroxy-4-androstene-3, 17-dione
Cylindrocarpon radicicola
Aspergillus & Penicillium sp.
Gliocladium catenulatum
Aspergillus & Rhizopus
Dactylium dendroides
lydroxy-
sterone
Streptomyces lavendulae
Fusarium sp.
Δ1,4-Androstadiene-3, 17-dione
Mucorales
Aspergillus sp.
Dactylium dendroides
Gliocladium, Aspergillus,
Penicillium, Fusarium sp.
4-Androstene-3, 17-dione
Progesterone
ihydroxy-
rone
Rhizopus nigricans
R. arrhizus
Streptomyces lavendulae
20β-Hydroxy-4-pregnene-3-one
Rhizopus sp.
Mucorales
Actinomycete
droxy-
one
11α-Hydroxyallopregnane-3, 20-dione
14α-Hydroxyprogesterone
16α-Hydroxypregnane-3, 20-dione

Some steroids produced from progesterone by microbiological transfor-

6, for a period of 17 to 48 hr. Aeration and agitation are pro-
ing growth in shake flasks or in bottles or tanks. The tempera-
aintained at an optimum for the production of the desired

he growth of the microbe, a measured quantity of the steroid,
in a suitable solvent, such as alcohol, acetone, or propylene
added to the medium. An enzyme (or enzymes) secreted by the
cts upon the steroid and performs the desired transformation

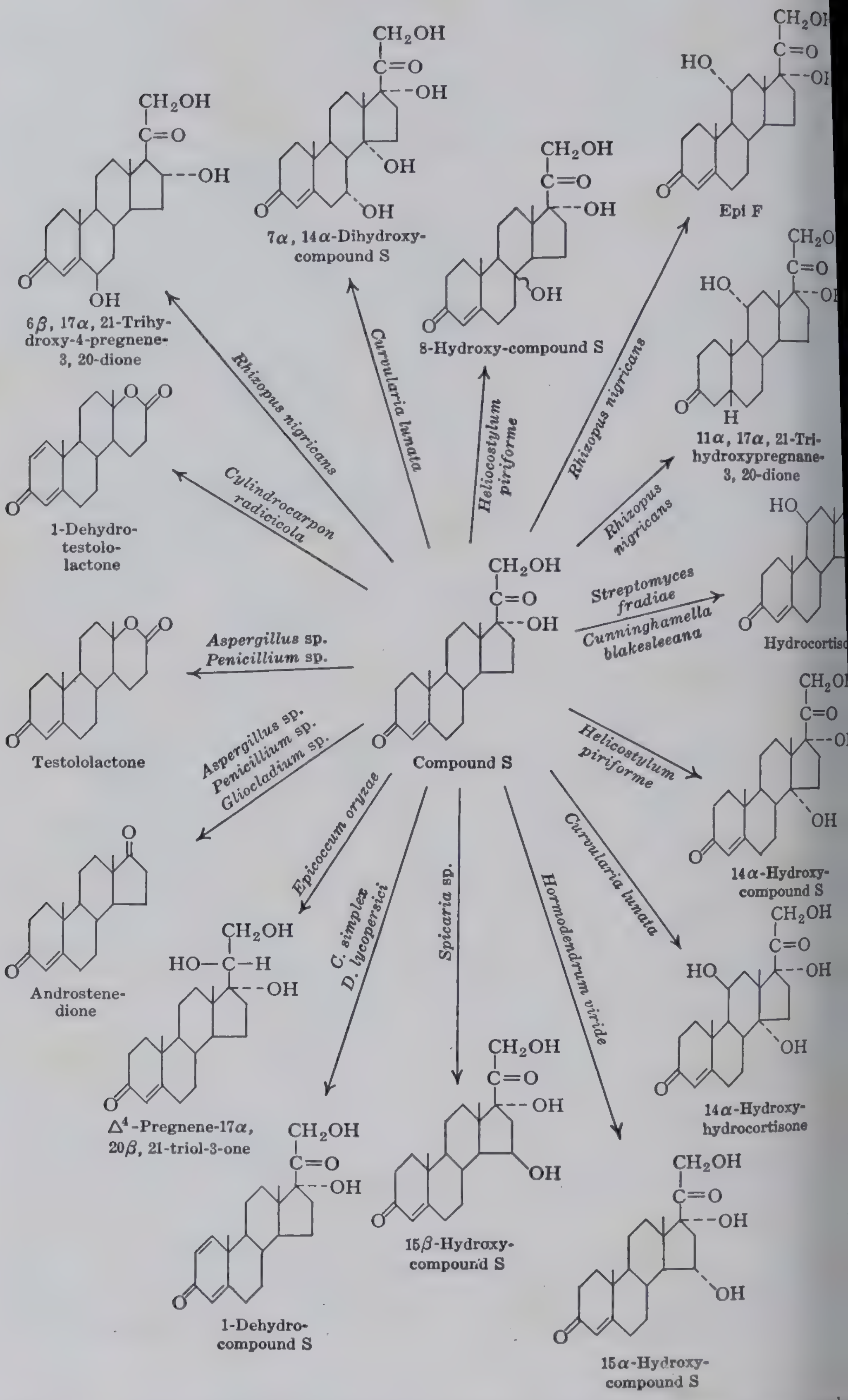

6β, 17α, 21-Trihydroxy-4-pregnene-3, 20-dione
7α, 14α-Dihydroxy-compound S
8-Hydroxy-compound S
Epi F
11α, 17α, 21-Trihydroxypregnane-3, 20-dione
1-Dehydrotestololactone
Hydrocortiso
Testololactone
Compound S
14α-Hydroxy-compound S
Androstenedione
14α-Hydroxyhydrocortisone
Δ4-Pregnene-17α, 20β, 21-triol-3-one
1-Dehydro-compound S
15β-Hydroxy-compound S
15α-Hydroxy-compound S
Rhizopus nigricans
Curvularia lunata
Heliocostylum piriforme
Rhizopus nigricans
Rhizopus nigricans
Cylindrocarpon radicicola
Streptomyces fradiae
Cunninghamella blakesleeana
Aspergillus sp.
Penicillium sp.
Aspergillus sp.
Penicillium sp.
Gliocladium sp.
Helicostylum piriforme
Epicoccum oryzae
C. simplex
D. lycopersici
Spicaria sp.
Hormodendrum viride
Curvularia lunata

Fig. 117. Some steroids which may be formed from Reichstein's compound
microbiological transformations.

6. SOME MEDIA USED FOR THE GROWTH OF FUNGI AND TRANSFORMATION OF STEROIDS

| | | | |
|---|---|---|---|
| of Peterson et al.* | | Media of Dulaney et al.† | |
| se, technical | 50 g. | Blackstrap invert molasses | 50 g. |
| e ‡ | 20 g. | Corn-steep liquor | 5 ml. |
| eep liquor | 3 g. | Water, distilled | 1 liter |
| tap, to make | 1 liter | | |
| th concentrated HCl). | 4.3–4.5 | Sucrose | 50 g. |
| | | $NaNO_3$ | 7.6 g. |
| ion of Dulaney et al.†, § | | $K_2HPO_4$ | 1 g. |
| e | 50 g. | $MgSO_4 \cdot 7H_2O$ | 0.5 g. |
| e | 20 g. | KCl | 0.5 g. |
| eep liquor | 5 ml. | $FeSO_4 \cdot 7H_2O$ | 40 mg. |
| distilled, to make | 1 liter | Water, distilled | 1 liter |
| of Dulaney et al. ¶ | | | |
| e | 50 g. | | |
| $HPO_4$ | 7.5 g. | | |
| $_4$ | 1 g. | | |
| | 0.5 g. | | |
| $7H_2O$ | 0.5 g. | | |
| $7H_2O$ | 0.01 g. | | |
| $7H_2O$ | 0.01 g. | | |
| distilled, to make | 1 liter | | |

---

Peterson et al., *Jour. Am. Chem. Soc.*, **74**: 5933 (1952). Copyright 1952 by ican Chemical Society and reprinted by permission of the copyright owner.
growth of Mucorales (*Rhizopus* species, in particular).
Dulaney et al., *Appl. Microbiol.*, **3**: 336 (1955). Used for the growth of *Aspergillus* and *Penicillium* and hydroxylation of progesterone and 11-hydroxycorticosterone and for the growth of *Dactylium dendroides* Fries in shake flasks.
zymatic digest of lactalbumin (Sheffield Farms, New York).
edia of Dulaney and associates adjusted to 6.5 before sterilization for 15 p.s.i.
ey et al., *op. cit.*, p. 372. Used for large-scale growth of *D. dendroides* and of 11α,17α-dihydroxyprogesterone.

ntrolled conditions of temperature, aeration, agitation, pH, and e usual transformation period is 24 to 48 hr.; however, it may be nder certain conditions.

nicrobial growth is separated from the fermentation liquor after nsformation and extracted with a suitable solvent, such as ne chloride, ethylene chloride, or chloroform. The extracts are liquor, and the mycelium (if a mold) is thrown away. The liquor added extract is extracted with a solvent, purified, and examined. ternate method, the beer may be extracted three to four times solvent (Eppstein et al., 1956).

**ples.** Peterson et al. (1952) used the following general method: of their medium was sterilized for 1 hr. at 120°C. in a 5-gal. ooled, and inoculated with the spores from a culture 1 week old he vegetative growth of the mold, usually *Rhizopus nigricans* or

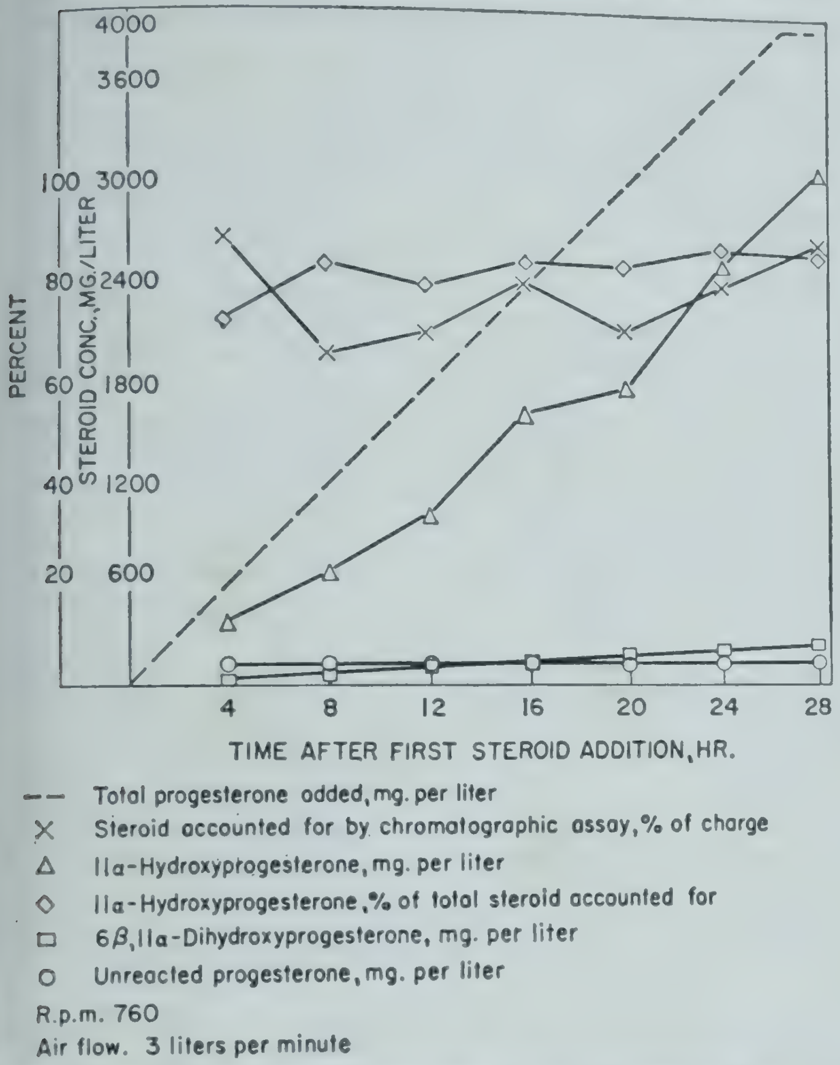
*R. arrhizus.* The medium was agitated with a paddle at about 2
and aerated with approximately 1 volume of air per minute. T
perature was held at 28°C. The mold was grown for 24 hr. or long
steroid, dissolved in acetone, ethanol, or other solvent, was adde
agitated medium and allowed to undergo transformation during
48-hr. period. The amount of steroid used generally varied from
1.0 g. per liter of medium. Methylene chloride and other solven
used for extracting the transformed steroid from the fermentation

Dulaney et al. (1955*b*) employed the following method for hydr
ing progesterone and 11-desoxy-17-hydroxycorticosterone in
Erlenmeyer flasks containing 50 ml. of glucose-Edamine medium, m
medium, or synthetic medium: The sterilized medium in flasks was
lated with a suspension of spores of a species of *Aspergillus* or *Peni*
and incubated at 28°C. for 24 to 48 hr. on rotary shakers rotating
r.p.m. and describing a circle with a diameter of 1.5 in. Ten
steroid dissolved in 2.5 ml. of propylene glycol was added to each
Incubation on the shakers was then continued for 24 hr. in order to
monohydroxylations. Longer periods were used when polyhydroxyl
were desired. After hydroxylation, the entire amount of broth was
minuted in a Waring Blendor for 1 min. and then extracted wit
25-ml. portions of chloroform for assay purposes or with three 50-ml
tions of the same solvent for quantitative determinations. The com
portions of extract for assay were evaporated to dryness at room tem
ture. The dried extract was dissolved in 2 ml. of pure methanol
assayed by the chromatographic methods of Zaffaroni et al. (1950, 1

**Effects of Variables on Transformations.** There is much to be lea
concerning the effects of variables on the efficacy of steroid transfo
tion. The report by Karow and Petsiavus (1956) on the effect of phy
variables on the microbiological transformation of progesterone and
pound S by *A. ochraceus* MF 348 (NRRL 405) provided a numbe
significant facts. Increased mechanical agitation of the conver
medium (2 per cent Edamine, 5 per cent dextrose, and 0.63 per cent
steep liquor, with a pH of 6.5 before sterilization) after addition of
steroid increased the rate of conversion owing to its effect on oxygen tr
fer and more efficient mixing. The rate of air flow was of large sig
cance, but no gross differences were observed in aeration rates of 0.5,
and 3.0 liters per min. per 3.4 liters of medium in 5-liter fermentors.
effect of temperature on the rate of conversion of progesterone in the ra
of 28 to 37°C. was not critical, nor was the time of adding the ste
between 18 and 48 hr.

The semicontinuous addition of the steroid was found to be desira
because it minimized the toxicity caused by the addition of a large amo
of solvent at one time, reduced the loss of steroid in the foam, retar
secondary conversion (11α-hydroxyprogesterone to 6β,11α-dihydroxy
gesterone), and made it possible to terminate the reaction more ac
rately. Results obtained by Karow and Petsiavus by adding progester
semicontinuously are shown in Fig. 118.

Dulaney, Stapley, and Hlavac (1955) showed that the 6β-hydroxylat



Hydroxylation of semicontinuously added progesterone. [*Courtesy of*
*ow and D. N. Petsiavus, Ind. Eng. Chem.*, **48**: 2213 (1956). *Copyright* 1956
*merican Chemical Society and reprinted by permission of the copyright*

α-hydroxyprogesterone produced from progesterone by *A. ochra-*
ld be prevented by a $Zn^{++}$ deficiency. They believed that the
or this was that there was not enough free $Zn^{++}$ for further pro-
thesis and the adaptive enzyme necessary for the 6β-hydroxyla-
not formed.

## TYPES OF TRANSFORMATIONS BROUGHT ABOUT BY MICROORGANISMS

nber of rather distinctive types of reactions involving steroids
performed by microorganisms. These have been classified by
(1956) and Eppstein et al. (1956) as simple, mixed, and mis-
is reactions. Table 207 indicates some of the simple and mixed
carried out by microbes.

**oxylation Reactions.** Hydroxylation, or oxygenation, of the
ccurs most frequently as a monohydroxylation, but dihydroxyla-

Table 207. Some Types of Reactions Brought about by Microbes

Simple Reactions

- Oxidation:
  - Hydroxylations or oxygenations:
    - Monohydroxylation
    - Dihydroxylation
    - Ketone formation
  - Dehydrogenation:
    - Oxidation of ring hydroxyl group
    - Formation of 1,4-dien-3-one system
    - Introduction of double bond in ring B
  - Rupture of carbon-to-carbon linkages:
    - Partial side-chain degradation
    - Side-chain oxidation with ketone or hydroxyl group formation
    - Ring D rupture with lactone formation
    - Ring A rupture with acid formation
- Reduction:
  - Ring double bonds
  - Carbonyls:
    - Ring ketones
    - Side chain

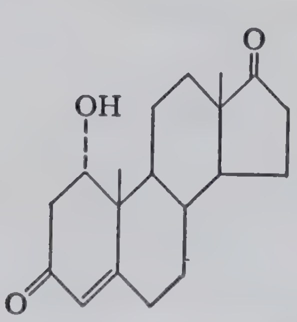
Mixed Reactions

- Hydroxylation:
  - With reduction of:
    - Ring double bonds
    - Carbonyl groups
  - With oxidation of:
    - Preexisting hydroxyl groups
  - With side-chain degradation
- Rupture of carbon-to-carbon lin
  - Oxidation of the side chain:
    - With ring hydroxylation
    - With oxidation of preexistin hydroxyl groups
    - With formation of 1,4-dien-3 system
      - With lactone formation in
      - With formation of 17β-hydr 17-ketone group
- Formation of a 1,4-dien-3-one c phore with other reactions
- Reduction reactions:
  - Reduction of a ring double bon ring ketone
  - Oxidation of a hydroxyl and re of a ketone group

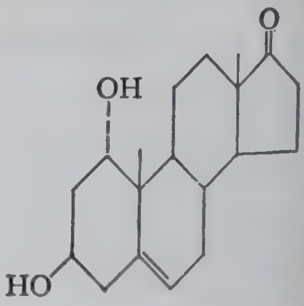
* Adapted from D. H. Peterson, *Record Chem. Progr., Kresge-Hooker Sci. Li* 211 (1956); and S. H. Eppstein et al., *Vitamins and Hormones,* **14**: 359 (1956).

tion is also common. Monohydroxylation of the steroid may take pla the 1α-, 6β-, 7α-, 7β-, 8- or 9-, 10-, 11α-, 11β-, 14α-, 15α-, 15β-, 17α-, or 21-carbon position. Dihydroxylation may occur in particul the 6β- and 11α-, 6β- and 14α-, 6β- and 17α-, 7β- and 11α-, 7α- and and 11α- and 17α-carbon positions. Many examples of monohydroxyl and a number of examples of dihydroxylation are given in the fo ing section.

*1α-Hydroxylation.* 1α-Hydroxy-4-androstene-3,17-dione may be pared by allowing a species of *Penicillium* to act on 4-androstent- dione (Dodson et al., 1957). Likewise, the same steroid may be prep from dehydroepiandrosterone, along with 1α-hydroxydehydroepian terone.

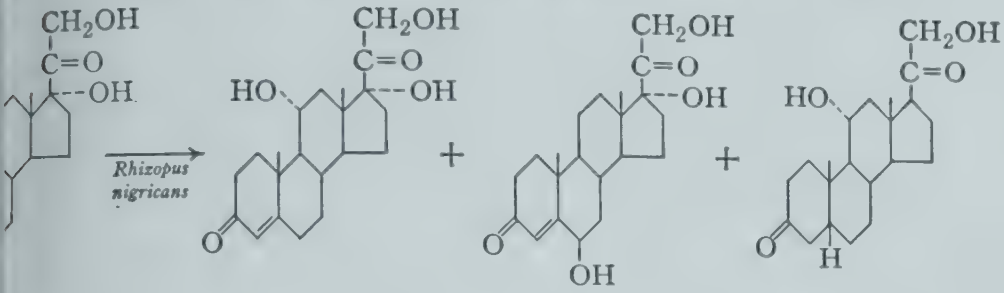


1α-Hydroxy-4-androstene-3, 17-dione



1α-Hydroxydehydroepiandrosterone

eroid may be transformed in more than one way by a given strain icrobe. Peterson and associates (1953*a*) found, for example, that tein's compound S may be transformed in 60 to 80 per cent yield -F, 5 to 10 per cent yield to 6β,17α,21-trihydroxy-4-pregnene-3,20- and traces to 11α,17α,21-trihydroxypregnane-3,20-dione.



und S

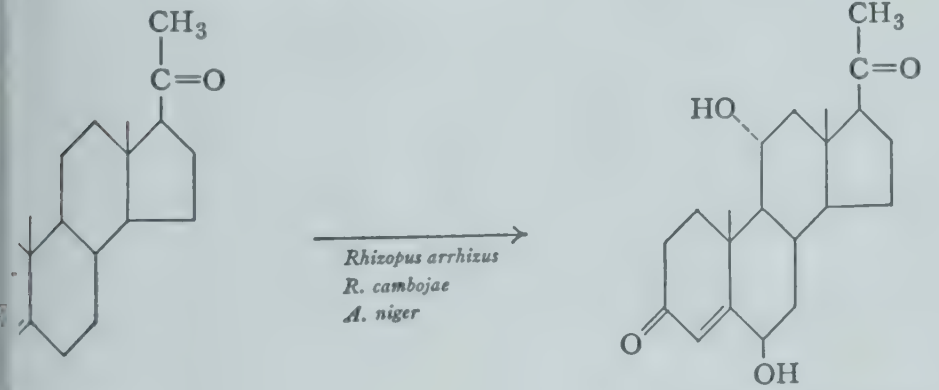
**11α, 17α, 21-Trihydroxy-4-pregnene-3, 20-dione (Epi F)**

**6β, 17α, 21-Trihydroxy-4-pregnene-3, 20-dione**

**11α, 17α, 21-Trihydroxy-pregnene-3, 20-dione**

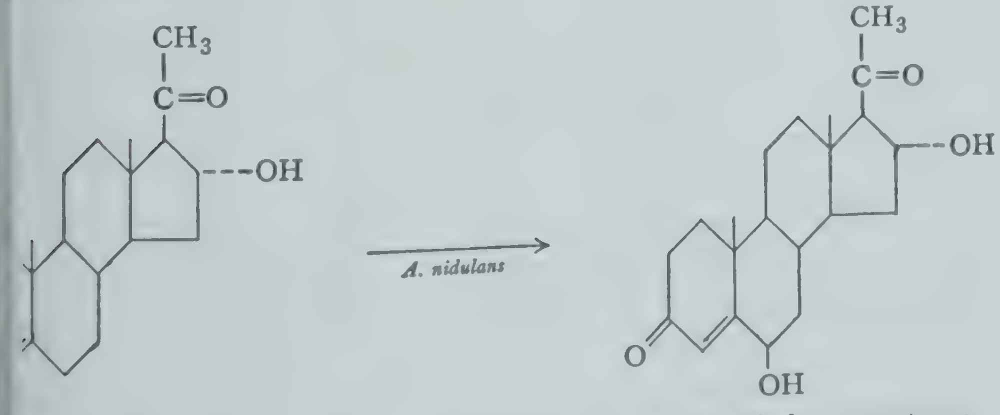
*ydroxylation.* β-Hydroxylation of the carbon-6 atom is brought y a number of molds. Other reactions often accompany the intro- of the 6β-hydroxyl group. However, the presence of another yl group in the molecule apparently inhibits hydroxylation in posi- ther than the 6-position. Some 6β-hydroxylations of 3-keto-$\Delta^4$- , including $C_{21}$, $C_{19}$, $C_{18}$, and $C_{22}$ compounds, are listed in 08.

ples of 6β-hydroxylations follow.



**Progesterone**

**6β, 11α-Dihydroxyprogesterone**

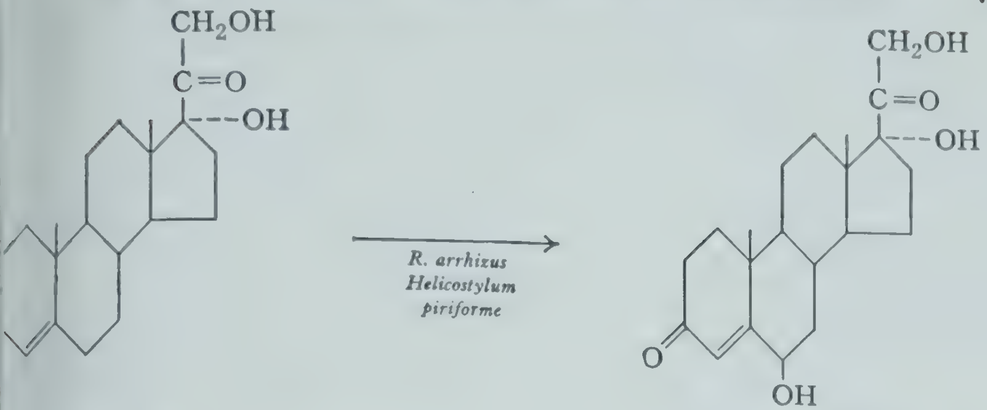


**Hydroxyprogesterone**

**6β, 16α-Dihydroxyprogesterone**

TABLE 208. SOME 6β-HYDROXYLATIONS

| Substrate | Product formed | Microorganism affecting transformation | Reference |
|---|---|---|---|
| Progesterone ................ | 6β,11α-dihydroxyprogesterone | *Rhizopus arrhizus*<br>*R. cambojae*<br>*Aspergillus niger*<br>*A. chevalieri* | Peterson et al. (1952).<br>Camerino et al. (1954).<br>Fried et al. (1952).<br>Dulaney et al. (1955*b*). |
| 16α-Hydroxyprogesterone ..... | 6β,11α-dihydroxyprogesterone | *A. nidulans* | Fried et al. (1955). |
| 17α-Hydroxyprogesterone ..... | 6β,17α-dihydroxyprogesterone | *R. arrhizus* | Meister et al. (1953*b*). |
| Deoxycortisol ............... | 6β,17α,21-trihydroxy-4-pregnene-3,20-dione | *R. arrhizus* | Murray and Peterson (1952).<br>Peterson et al. (1953*a*). |
| | | *Helicostylum piriforme*<br>*Trichothecium roseum* | Meister et al. (1953*d*).<br>Meister et al. (1953*d*). |
| 11-Deoxycorticosterone ....... | 6β-hydroxy-11-deoxycorticosterone | *R. arrhizus*<br>*Streptomyces fradiae*<br>*T. roseum*<br>*Lenzites abientina* | Eppstein et al. (1953*a*).<br>Haines and Colingsworth (1953).<br>Meystre et al. (1954).<br>Wettstein et al. (1955). |
| 4-Androstene-3,17-dione ....... | 6β-hydroxy-4-androstene-3,17-dione | *R. arrhizus*<br>*R. nigricans*<br>*R. reflexus* | Eppstein et al. (1954). |
| | | *A. niger* | Fried et al. (1955). |
| Testosterone ................ | 6β-hydroxytestosterone | *R. arrhizus*<br>*R. nigricans*<br>*R. reflexus* | Eppstein et al. (1954). |
| 17α-Methyltestosterone ....... | 6β-hydroxy-17α-methyltestosterone | *R. arrhizus*<br>*R. nigricans*<br>*R. reflexus* | Eppstein et al. (1954). |
| 19-Nortestosterone .......... | 6β-hydroxy-19-nortestosterone | *R. nigricans* | |
| 3-Ketobisnor-$\Delta^4$-cholene-22-al... | | | |



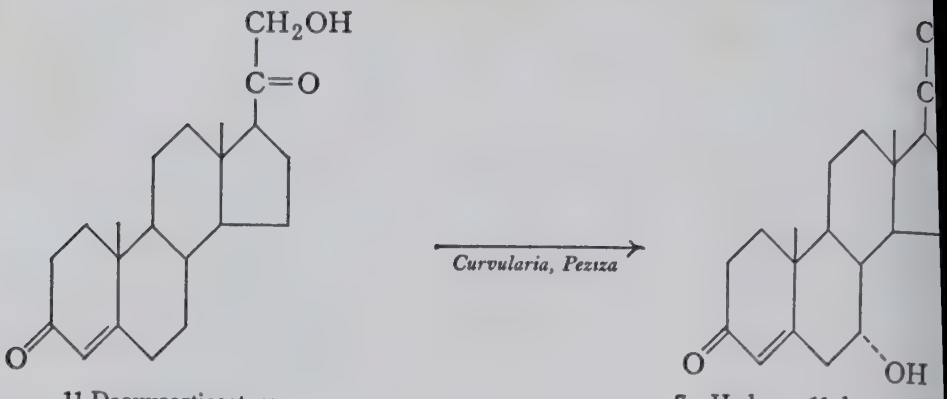
Compound S

6β-Hydroxy-compound S

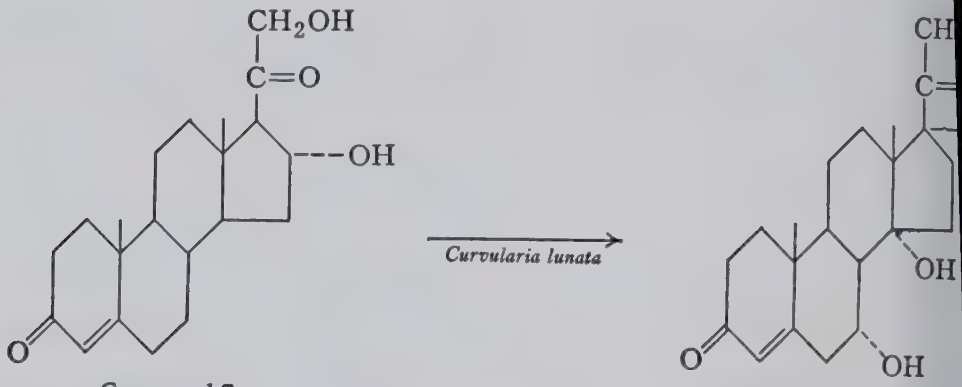
*ydroxylation.* The steroid nucleus may be hydroxylated in the 7β-positions by a number of microorganisms, as may be ascertained erence to Table 209 and to some of the following examples.

TABLE 209. SOME 7-HYDROXYLATIONS

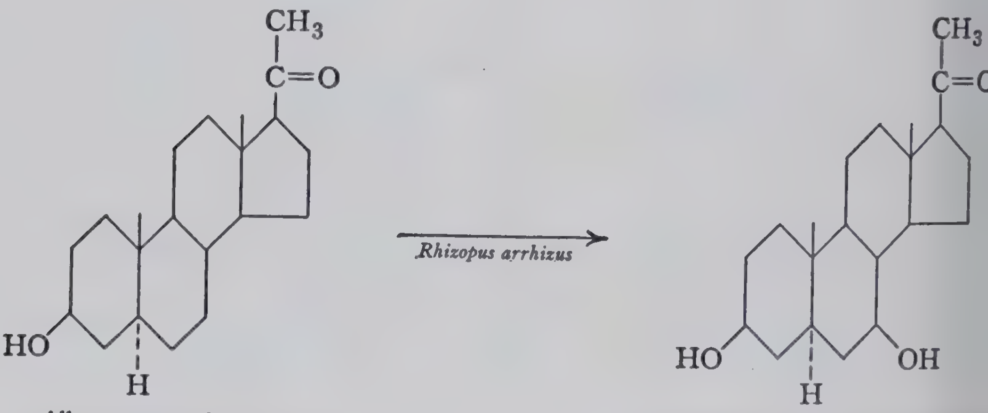
| bstrate | Product formed | Microorganism affecting transformation | Reference |
|---|---|---|---|
| terol ..... | 7ξ-hydroxy-cholesterol | *Proactinomyces roseum* | Krámli and Horváth (1948). |
| ihydroxy-<br>egnane-<br>e ....... | 3β,7β,21-trihydroxy-allopregnane-20-one | *Rhizopus* sp. | Kahnt et al. (1952). |
| oxyallo-<br>ane-20-<br>......... | 3β,7β-dihydroxy-allopregnane-20-one | *R. arrhizus* | Murray and Peterson (1952). |
| olone ... | 3β,7β,11α-trihydroxy-5-pregnene-20-one and<br>3β,11α-dihydroxy-5-pregnene-7,20-dione | *R. arrhizus* | Murray and Peterson (1954). |
| erone ... | 7-progesterone | *Phycomyces blakesleeanus* | Fried et al. (1955). |
| y-<br>sterone.. | 7α-hydroxy-11-deoxy-corticosterone | *Peziza* sp.<br>*Curvularia* sp. | Meystre et al. (1955). |
| nd S ... | 7α,14α-dihydroxy-compound S | *C. lunata* | Agnello et al. (1956). |


Curvularia, Peziza

11-Deoxycorticosterone 7α-Hydroxy-11-deoxycortic

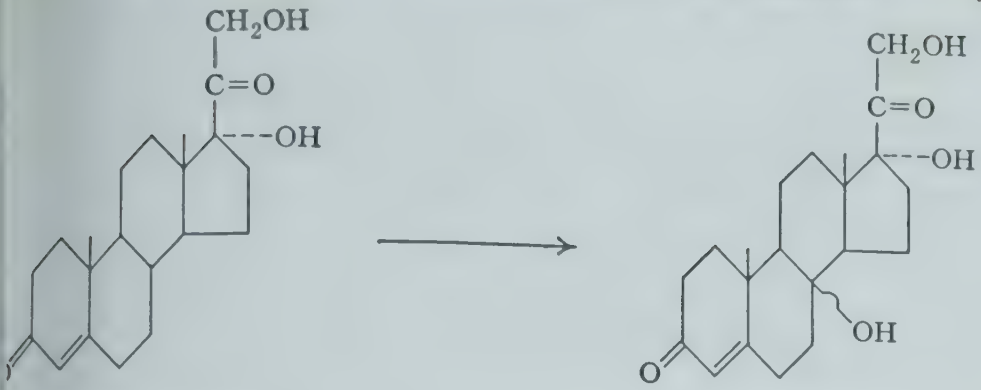

Curvularia lunata

Compound S 7α, 14α-Dihydroxy-compou


Rhizopus arrhizus

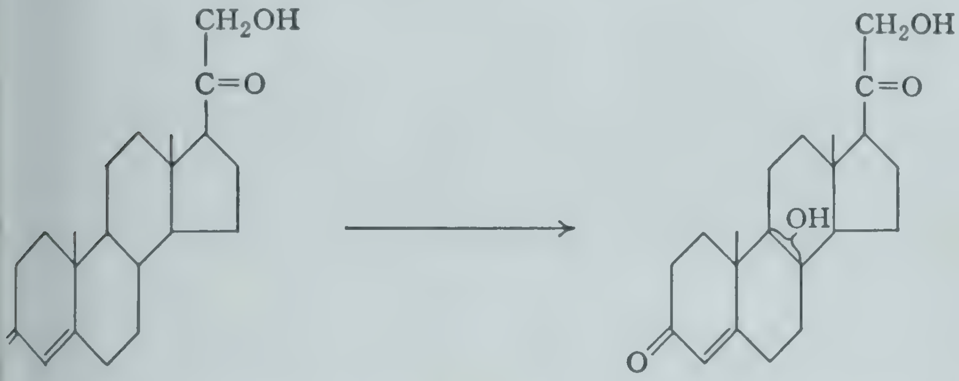
Allopregnane-3β-ol-20-one Allopregnane-3β, 7β-diol-20-one

*8-(or 9-)Hydroxylation.* Considerable difficulty has been exper in assigning a position and configuration to the hydroxyl group ad the 8- or 9-position. Murray and Peterson (1952) reported that stein's compound S was hydroxylated in the 8-position by *H stylum piriforme,* as indicated below, and that 14α-compound S wa formed:



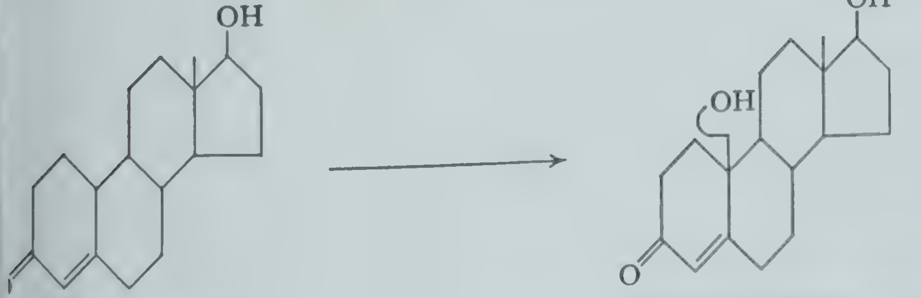
**Reichstein's compound S** **8-Hydroxy-compound S**

ree different groups of scientists observed a hydroxylation of deoxy- ›osterone at either the 8- or the 9-position. Wettstein (1955) used *ılaria pallescens;* Meister (1955), *Mucor parasiticus;* and Stone and iates (1955), *Neurospora crassa.*



d et al. (1955) found that 8- (or 9-) hydroxyprogesterone and lroxyprogesterone were formed when progesterone was incubated *treptomyces aureofaciens.*

*Iydroxylation.* Pederson and his associates (1956) reported that *ıus nigricans* transformed 19-nortestosterone to 10-hydroxy-19- -osterone. The configuration of the hydroxyl group was not shed.



**19-Nortestosterone** **10-Hydroxy-19-nortestosterone**

*ydroxylation.* The 11-carbon atom of the steroid nucleus may be ›lated in the alpha or beta configuration by means of micro- ms. The 11α-hydroxyl group was first introduced into progesterone ls of the order Mucorales by Peterson and Murray (1952). This

Table 210. Examples of 11$\alpha$-Hydroxylations Accomplished by Molds

| Substrate | Product formed | Microorganism concerned | Yield (approx.), % | Reference |
|---|---|---|---|---|
| Progesterone ..................... | 11$\alpha$-Hydroxyprogesterone | *Rhizopus arrhizus* | 10 | Peterson and Murray (1952). |
| | | *R. nigricans* | 80–90 | Peterson et al. (1952*a*). |
| | | *Rhizopus* sp. | 45 | Mancera et al. (1952). |
| | | *Rhizopus* sp. | 40 | Kahnt et al. (1952). |
| | | *Aspergillus niger* | 35 | Fried et al. (1952). |
| | | *Pestalotia foedans* | ..... | Shull et al. (1954). |
| | | *Erotium chevalieri* | ..... | Shull et al. (1955). |
| | | *A. niger* | 85 | Dulaney et al. (1955*a*). |
| | | *A. ochraceus* | 64 | Dulaney et al. (1955*a*). |
| | | *A. amstelodami* | 64 | Dulaney et al. (1955*a*). |
| | | *A. pseudoglaucus* | 64 | Dulaney et al. (1955*a*). |
| | | *A. repens* | 63 | Dulaney et al. (1955*a*). |
| | | *Dactylium dendroides* | 30 | Dulaney et al. (1955*b*). |
| | 6$\beta$,11$\alpha$-Dihydroxyprogesterone | *R. arrhizus* | 7.5 | Peterson et al. (1952*a*, *b*). |
| | | *A. niger* | 20 | Fried et al. (1952). |
| | 11$\alpha$,17$\alpha$-Dihydroxyprogesterone | *Aspergillus* sp. | | Dulaney et al. (1955*a*). |
| | | *D. dendroides* | | Dulaney et al. (1955*b*). |
| Pregnanedione .................. | 11$\alpha$-Hydroxypregnanedione | *R. nigricans* | 40 | Eppstein et al. (1953*b*). |
| Allopregnanedione ............. | 11$\alpha$-Hydroxyallopregnanedione | *R. nigricans* | 25 | Eppstein et al. (1953*b*). |
| 6-Dehydroprogesterone .......... | 11$\alpha$-Hydroxy-6-dehydroprogesterone | *R. nigricans* | 60 | Peterson et al. (1953*b*). |
| 16-Dehydroprogesterone ........ | 11$\alpha$-Hydroxy-16-dehydroprogesterone | *R. nigricans* | 30 | Meister et al. (195 |

| | | concerned | | Reference |
|---|---|---|---|---|
| 17α-Hydroxyprogesterone | 11α,17α-Dihydroxyprogesterone | *A. niger* | 15–25 | Fried et al. (1952). |
| | | *R. nigricans* | 75 | Meister et al. (1953*b*). |
| | | *D. dendroides* | 27 | Dulaney et al. (1955*b*). |
| Deoxycorticosterone | Epicorticosterone | *A. niger* | 67 | Fried et al. (1952). |
| | Epicorticosterone | *Rhizopus* sp. | ..... | Kahnt et al. (1952). |
| | Epicorticosterone | *R. nigricans* | 55 | Eppstein et al. (1953*a*). |
| | Epicorticosterone | *Dactylium dendroides* | ..... | Dulaney et al. (1955*b*). |
| | Epi-F | *Cephalothecium roseum* | ..... | Meister et al. (1954). |
| | Epi-F | *D. dendroides* | ..... | Dulaney et al. (1955*b*). |
| Compound S | Epi-F | *A. niger* | 25 | Fried et al. (1952) |
| | Epi-F | *A. nidulans* | 70 | Fried et al. (1955). |
| | Epi-F | *R. nigricans* | 60 | Peterson et al. (1953). |
| | Epi-F | *Rhizopus* sp. | 30 | Kahnt et al. (1952). |
| 4-Androstene-3,17-dione | 11α-Hydroxy-4-androstene-3,17-dione | *Rhizopus* sp. | ..... | Eppstein et al. (1954). |
| Testosterone | 11α-Hydroxytestosterone | *Rhizopus* sp. | ..... | Eppstein et al. (1954). |
| 17α-Methyltestosterone | 11α-Hydroxy-17α-methyltestosterone | *Rhizopus* sp. | ..... | Eppstein et al. (1954). |
| 3-Ketobisnor-4-cholene-22-al | 3-Ketobisnor-4-cholene-11α,22-diol | *R. nigricans* | 20–40 | Meister et al. (1954). |
| 19-Nortestosterone | 11α-Hydroxy-19-nortestosterone | *R. nigricans* | ..... | Pederson et al. (1956). |
| 16α,17α-Oxidoprogesterone | 11α-Hydroxy-16α,17α-oxidoprogesterone | *R. nigricans* | ..... | Peterson et al. (1955). |

accomplishment marked a most significant advance in the us organisms to perform transformations which were difficult to c chemical means.

Tables 210 and 211 give examples of 11$\alpha$-hydroxylation

TABLE 211. EXAMPLES OF SOME 11$\beta$-HYDROXYLATIONS ACCOMPLIS BY MICROORGANISMS

| Substrate | Product formed | Microorganism concerned | Refe |
|---|---|---|---|
| Compound S .... | Hydrocortisone | *Streptomyces fradiae* | Colingswor (1952). |
| | | *Cunninghamella blakesleeana* | Hanson et |
| | | *Curvularia* sp. | Shull et al. |
| | | *Coniothyrium* sp. | Fried et al. |
| | | *C. lunata* | Shull and |
| | | *Pycnosporium* sp. | Davisson e 1956). |
| | | *Rhodoseptoria* sp. | Kita (Shull |
| | | *Dothichiza* sp. | Kita and S 1956). |
| | 14$\alpha$-Hydroxyhydrocortisone | *C. lunata* | Agnello et |
| 14$\alpha$,15$\alpha$-Epoxido-compound S... | 11$\beta$-Hydroxy-14$\alpha$,15$\alpha$-Epoxido-compound S | *C. lunata* | Bloom et 1956). |
| 11-Deoxycorticosterone ..... | Corticosterone | *Curvularia* sp. | Shull and K |
| | | *Cunninghamella blakesleeana* | Mann et al. |
| Progesterone ... | 11$\beta$-Hydroxyprogesterone | *C. lunata* | Shull and K |
| | Corticosterone | *C. lunata* | Rubin et al. |
| 17$\alpha$-Hydroxyprogesterone .. | 11$\beta$,17$\alpha$-Dihydroxyprogesterone | *Curvularia* sp. | Shull and K |
| | 11$\beta$,14$\alpha$,17$\alpha$-Trihydroxyprogesterone | *Curvularia* sp. | Shull et al. |

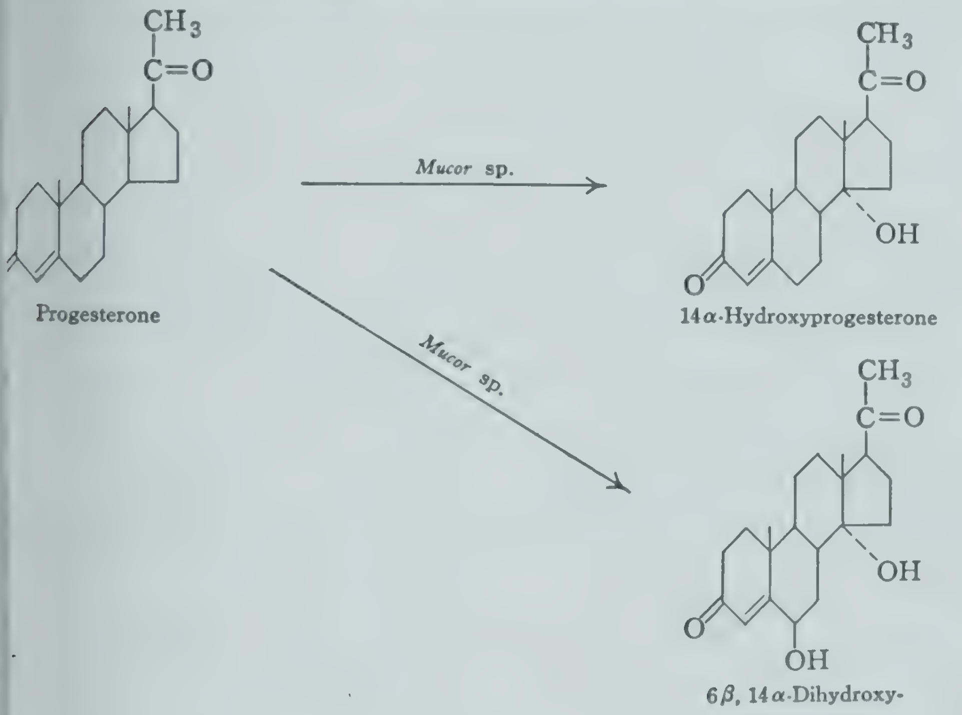
hydroxylation, respectively. It will be observed that the forme hydroxylation is more common than the latter.

Dulaney et al. (1955*a*) tested 475 cultures of aspergilli, rep 38 species, and found that the ability to transform progest 11$\alpha$-hydroxyprogesterone, and also Reichstein's compound S pound Epi-F, was common among them. *A. niger, A. amstelo*

*aceus, A. pseudoglaucus, A. repens,* and *A. unguis* were among those
to transform fairly large percentages (some up to 80 per cent) of
esterone to 11α-hydroxyprogesterone. A regular pattern appeared to
ollowed by cultures of the aspergilli in which progesterone was trans-
ned to 11α-hydroxyprogesterone and the latter to 6β-11α-dihydroxy-
esterone. In a few instances, the molds converted progesterone to
hydroxyprogesterone and then to 11α,17α-dihydroxyprogesterone.

ulaney and associates also examined 476 cultures, representing 92
es. of the genus *Penicillium*. Transformation of progesterone was
d to be of rare occurrence. *P. corylophilum, P. lilacinum,* and *P.*
*um* were the only cultures discovered which hydroxylated proges-
e at the 11 position. The maximum yield was 35 per cent.

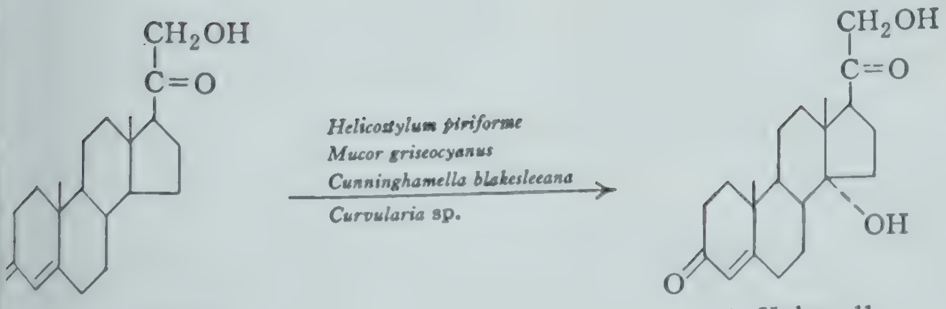
*-Hydroxylation.* Murray and Peterson (1952) gave an example of
ydroxylation by *Helicostylum piriforme* in the patent issued to them.
212 lists some examples of 14α-hydroxylation. Illustrations follow:


CH3
C=O
Mucor sp.
CH3
C=O
OH
O
Mucor sp.
CH3
C=O
OH
O
OH

Progesterone

14α-Hydroxyprogesterone

6β, 14α-Dihydroxyprogesterone


CH2OH
C=O
Helicostylum piriforme
Mucor griseocyanus
Cunninghamella blakesleeana
Curvularia sp.
CH2OH
C=O
OH
O

1-Deoxycorticosterone

14α-Hydroxy-11-deoxycorticosterone

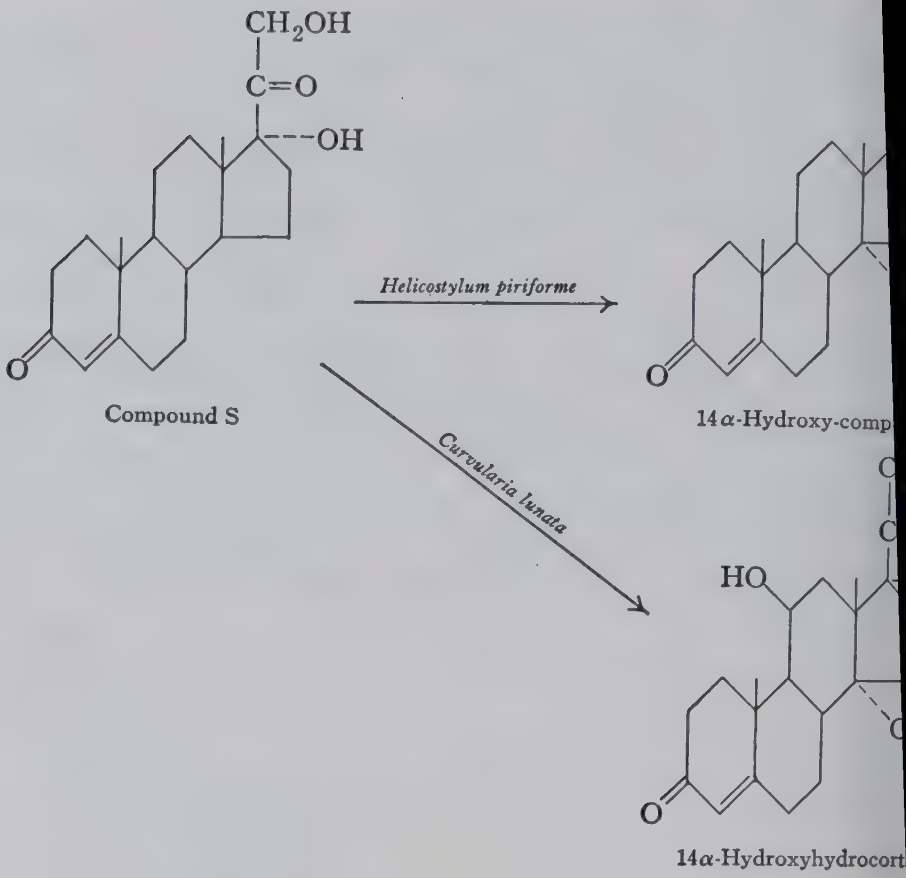

CH2OH
C=O
OH
O
Compound S
Helicostylum piriforme
Curvularia lunata
O
14α-Hydroxy-comp
HO
O
14α-Hydroxyhydrocort

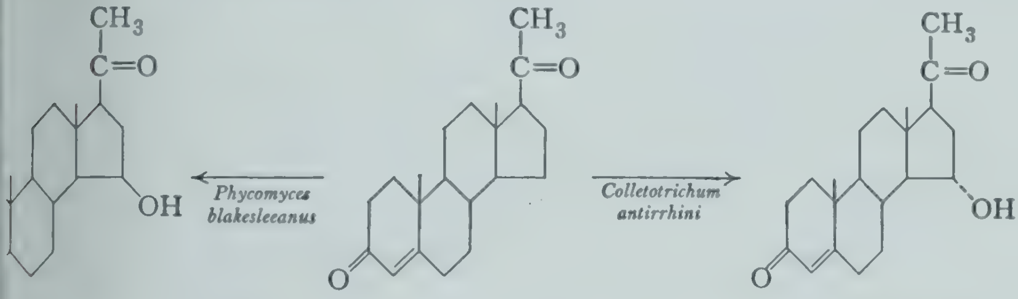
TABLE 212. SOME EXAMPLES OF 14α-HYDROXYLATIONS

| Substrate | Product formed | Microorganism concerned | Refer |
|---|---|---|---|
| 11-Deoxycorticosterone .... | 14α-Hydroxy-11-deoxycorticosterone | *Mucor griseocyanus*<br>*Helicostylum piriforme*<br>*Curvularia* sp.<br>*Cunninghamella blakesleeana* | Meister et<br>Meister et<br>Wettstein (<br>Mann et al. |
| Progesterone .... | 14α-Hydroxyprogesterone<br>6β,14α-Dihydroxyprogesterone | *Mucor* sp.<br>*Bacillus cereus*<br>*Mucor* sp. | Meister et a<br>Fried et al.<br>Camerino et |
| 17α-Hydroxyprogesterone .. | 11β,14α,17α-Trihydroxyprogesterone | *C. lunata* | Shull et al. |
| Compound S ... | 14α-Hydroxycompound S<br>14α-Hydroxyhydrocortisone | *H. piriforme*<br>*C. lunata* | Meister et a<br>Agnello et a |

TABLE 212. SOME EXAMPLES OF 14α-HYDROXYLATIONS—(*Continued*)

| Substrate | Product formed | Microorganism concerned | Reference |
|---|---|---|---|
| -Dehydro-<br>npound S . . | 14α-Hydroxy-$\Delta^{9(11)}$-dehydro-compound S | *C. lunata* | Bloom et al. (Shull, 1956). |
| sterone . . . . | 14α-Hydroxy-testosterone | *Mucor* sp. | Meister et al. (1953*d*). |

*-Hydroxylation.* The 15-carbon atom of the steroid may be hydroxy-
l with either the alpha or the beta configuration. Fried et al. (1955)
unced that progesterone was capable of being transformed as indi-
l in the following diagram:

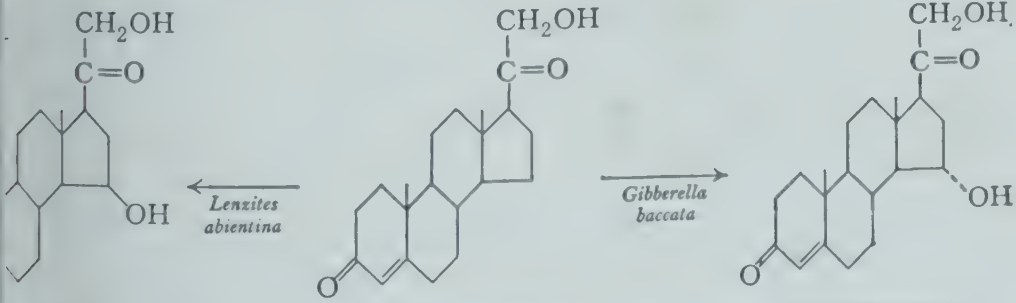


roxyprogesterone

Progesterone

15α-Hydroxyprogesterone

re and associates (1955) (refer also to Wettstein, 1955) showed
11-deoxycorticosterone could be converted to the 15α-hydroxy-
und with *Gibberella baccata* and to the 15β-hydroxy-compound
*enzites abientina:*

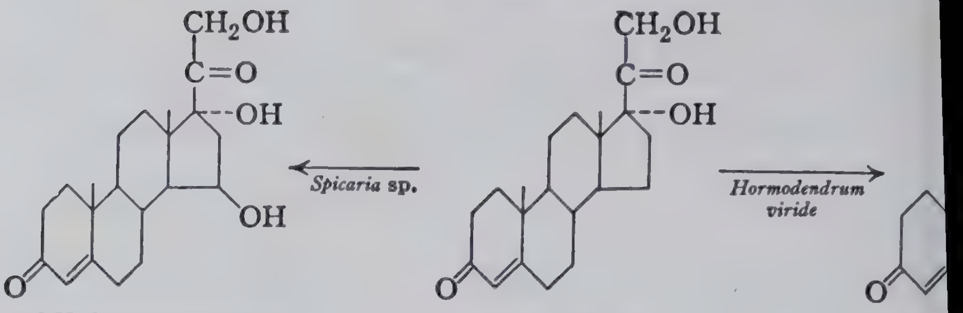


Hydroxy-11-
orticosterone

11-Deoxycorticosterone

15α-Hydroxy-11-
deoxycorticosterone

y and associates (1955*a*) reported that a number of species of
*lium* possessed the ability to form 15-hydroxyprogesterone, but the
ration was not given. Bernstein and coworkers (1956) observed
*ormodendrum viride* produced the 15α-hydroxy-compound and that
*a* species produced the 15β-hydroxy-derivative of compound S:

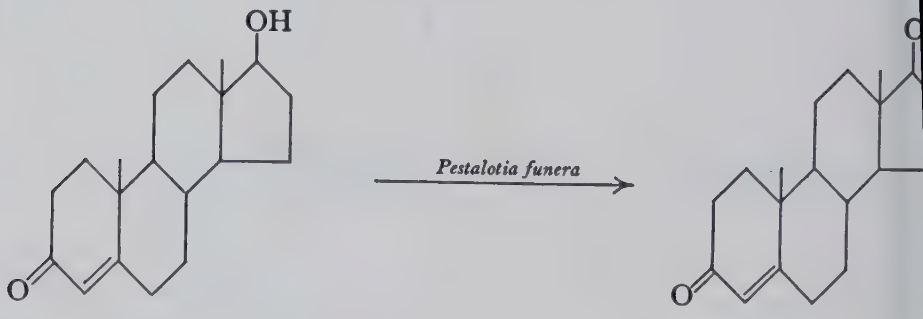

CH₂OH
C=O
OH
OH
O
Spicaria sp.
CH₂OH
C=O
OH
O
Hormodendrum viride
O

15β-Hydroxy-compound S

Compound S

15α-Hy

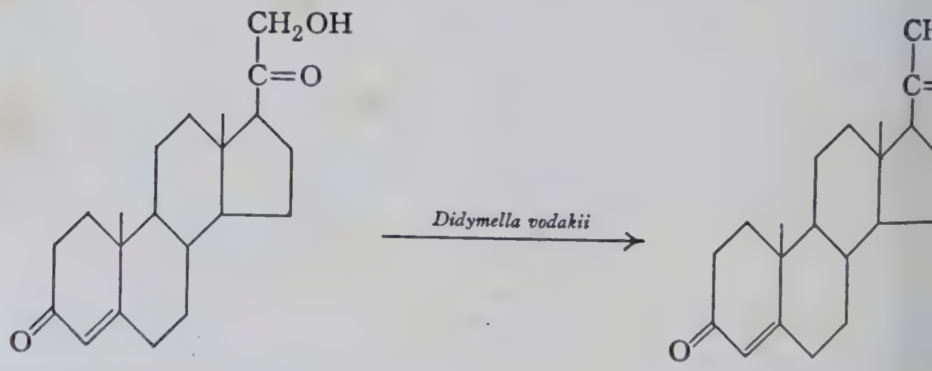
*16α-Hydroxylation.* Perlman and associates (1952) an
formation of 16α-hydroxypregnane-3,20-dione from progest
an actinomycete (refer to Fig. 116). Thoma and coworkers
complished the 16α-hydroxylation of progesterone with *S*
*roseochromogenus.* Vischer et al. (1954) observed that sever
*Streptomyces* possessed the ability to transform 11-deoxycorti
16α-hydroxy-11-deoxycorticosterone in about 20 per cent yi
and associates (1955) prepared 16α-hydroxytestosterone from t


OH
O
Pestalotia funera
O

Testosterone

16α-Hydroxytesto

Wettstein (1955) accomplished the transformation of 11-deo
terone to the 16α-derivative with *Didymella vodakii.*

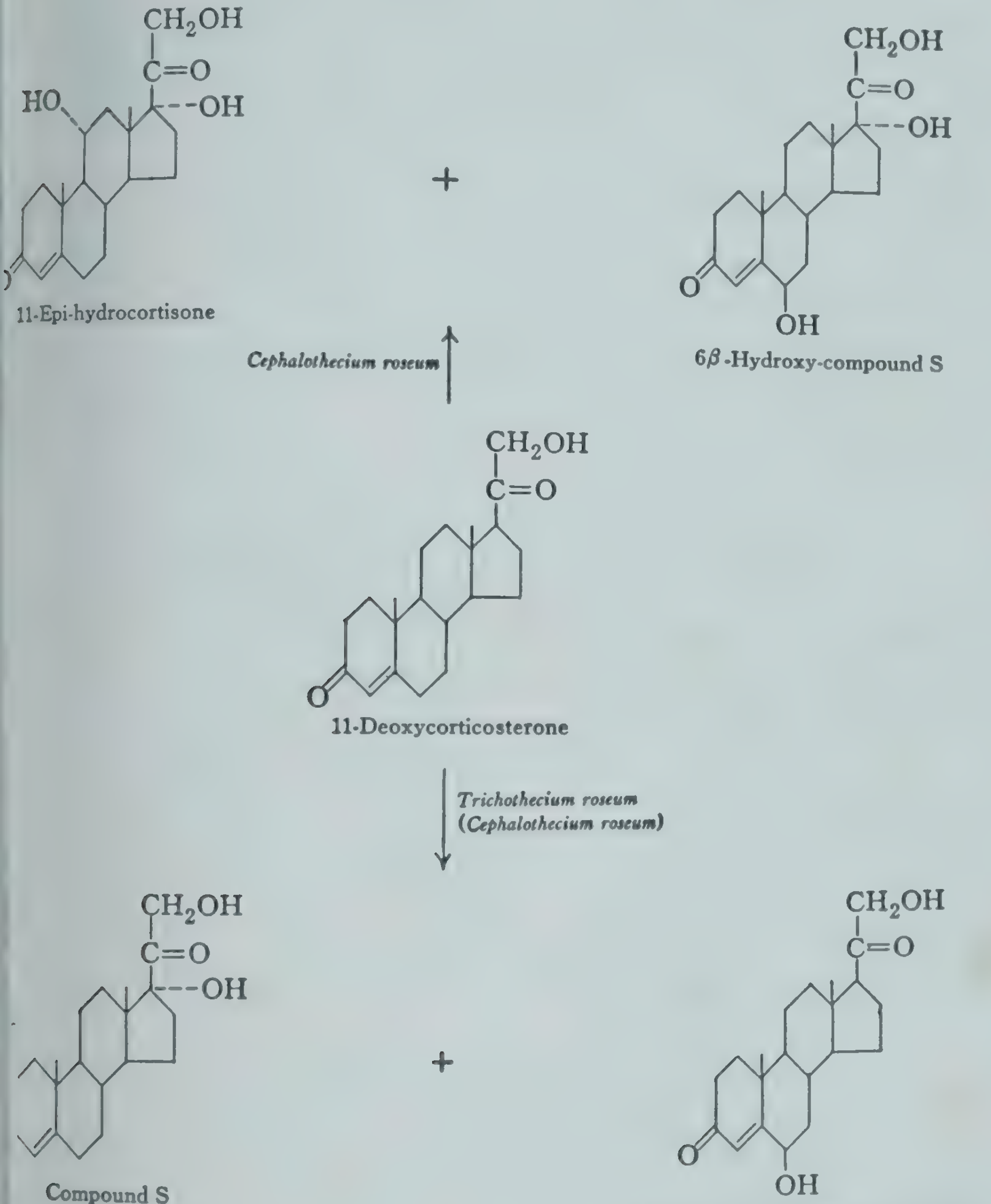

CH₂OH
C=O
O
Didymella vodakii
CH
C=
O

11-Deoxycorticosterone

16α-Hydroxy-11-
deoxycorticostero

*17α-Hydroxylation.* This is an important hydroxylation and
is difficult to obtain by chemical means (Peterson, 1955).
announcements relating to the availability of the microbiologic
were made nearly simultaneously by Meister and associates (1

Meystre and coworkers (1954), who used *Cephalothecium roseum* Cda T.C. 8685 and *Trichothecium roseum,*[1] respectively. These molds are arently identical, except possibly for strain differences. 17α-Hydroxyions made by these and other molds are listed in Table 213. Examples the hydroxylations follow.


11-Epi-hydrocortisone

6β-Hydroxy-compound S

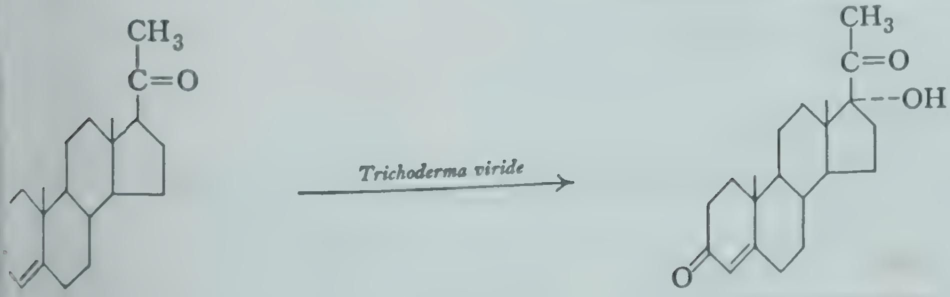
*Cephalothecium roseum*

11-Deoxycorticosterone

*Trichothecium roseum*
*(Cephalothecium roseum)*

Compound S

6β-Hydroxy-11-deoxycorticosterone

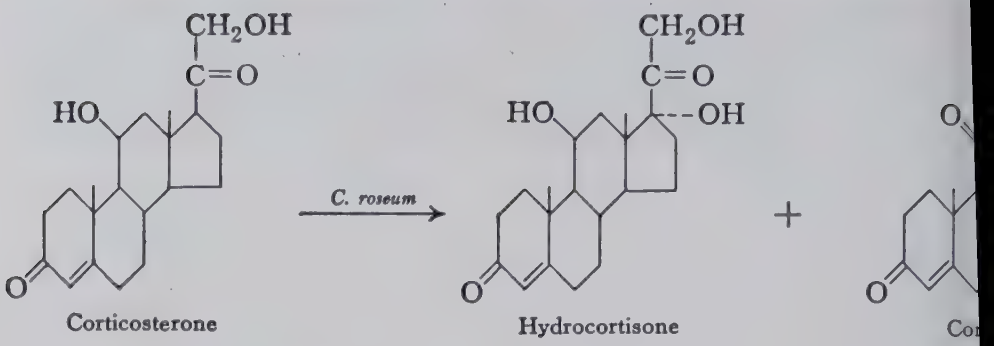

Progesterone

*Trichoderma viride*

17α-Hydroxyprogesterone

---

[1] correct genus name is *Cephalothecium.*



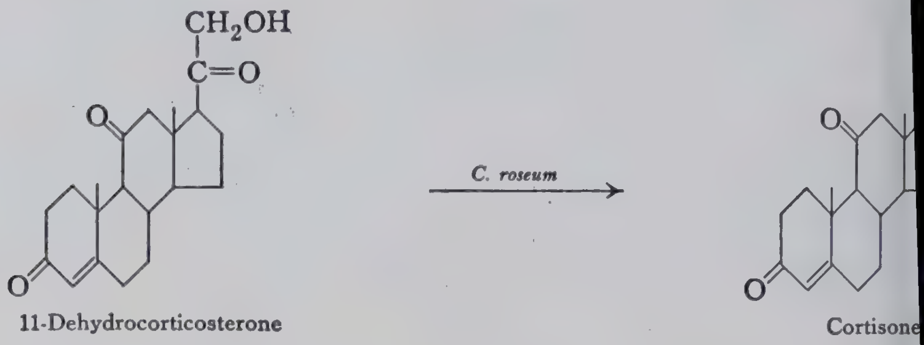
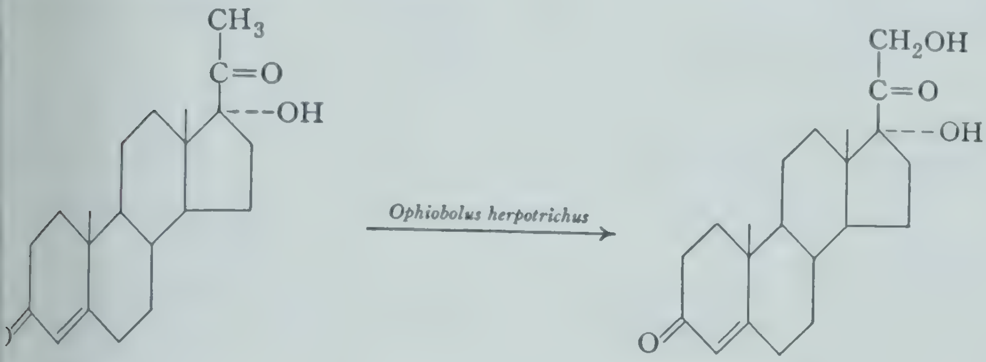


TABLE 213. SOME EXAMPLES OF 17α-HYDROXYLATIONS

| Substrate | Product formed | Microorganism concerned | Refere |
|---|---|---|---|
| Corticosterone . . | Hydrocortisone<br>Cortisone | *Cephalothecium roseum*<br>*C. roseum* | Meister et a<br>Meystre et a |
| 11-Dehydrocorticosterone . . . . . | Cortisone | *C. roseum*<br>*C. roseum* | Meister et a<br>Meystre et a |
| 11-Deoxycorticosterone . . . . . | 11-Epihydrocortisone<br>6β-Hydroxy-compound S<br>17α-Hydroxy-11-deoxycorticosterone<br>6β-Hydroxy-11-deoxycorticosterone | *C. roseum*<br>*C. roseum* | Meister et a<br>Meystre et a |
| Progesterone . . . . | 11α,17α-Dihydroxy-progesterone<br>17α-Hydroxy-progesterone | *C. roseum*<br>*Trichoderma viride* | Meister et al<br>McAleer and (1956). |
| 11α-Hydroxy-progesterone . . | 11α,17α-Dihydroxy-progesterone | *Aspergillus* sp.<br>*Dactylium dendroides* | Dulaney et al<br>Dulaney et al |

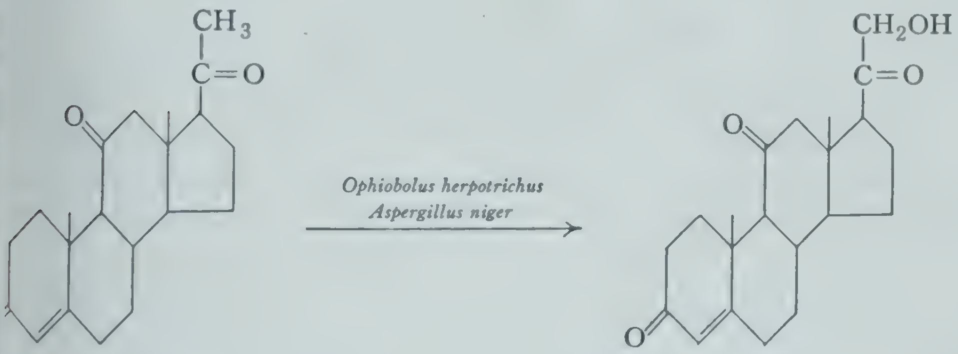
NOTE: 11-Epihydrocortisone = 11α,17α,21-trihydroxy-$\Delta^4$-pregnene-3,20-dion droxy-compound S = 6β,17α,21-trihydroxy-$\Delta^4$-pregnene-3,20-dione. Hydroc 17α-hydroxycorticosterone. Cortisone = 17α-hydroxy-11-dehydrocorticostero

*21-Hydroxylation.* Meystre and associates (1954) first reported the roduction of a 21-hydroxyl group by microbiological means, using *hiobolus herpotrichus*. Later (1955), Zaffaroni and collaborators indi-ed that it was possible to convert progesterone and a number of its ivatives to their corresponding 21-hydroxyl compounds through the use *A. niger*. McAleer and Dulaney (1956) and Rubin (1956) accom-hed similar transformations with *Wojnowicia graminis* and *C. lunata*. examples of 21-hydroxylations, the reader is referred to Table 214 to the illustrations which follow.



17α-Hydroxyprogesterone

Compound S



11-Ketoprogesterone

11-Dehydrocorticosterone

TABLE 214. SOME EXAMPLES OF 21-HYDROXYLATION

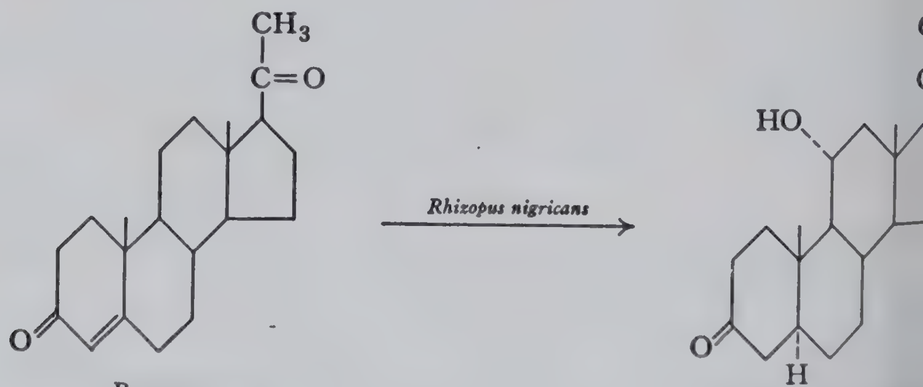
| bstrate | Product formed | Microorganism concerned | Reference |
|---|---|---|---|
| terone .... | 11-Deoxycorticos-terone | *Ophiobolus herpotrichus*<br>*Aspergillus niger* A.T.C.C. 9142<br>*Wojnowicia graminis* | Meystre et al. (1954).<br>Zaffaroni et al. (1955).<br>McAleer and Dulaney (1956). |
| | Corticosterone | *Curvularia lunata* | Rubin et al. (1956). |
| roxy-sterone .. | 6β-Hydroxy-11-deoxycorticosterone | *A. niger* A.T.C.C. 9142 | Zaffaroni et al. (1955). |

TABLE 214. SOME EXAMPLES OF 21-HYDROXYLATION—(*Continu*

| Substrate | Product formed | Microorganism concerned | Re |
|---|---|---|---|
| 11-Ketoprogesterone ..... | 11-Dehydrocorticosterone | *O. herpotrichus*<br>*A. niger* | Meystre e<br>Zaffaroni |
| 11α-Hydroxyprogesterone .. | 11-Epicorticosterone | *A. niger* A.T.C.C. 9142 | Zaffaroni e |
| 11β-Hydroxyprogesterone .. | Corticosterone | *A. niger* A.T.C.C. 9142 | Zaffaroni e |
| 19-Norprogesterone ..... | 19-Nor-11-deoxycorticosterone | *A. niger* A.T.C.C. 9142 | Zaffaroni et |

**Hydrogenation Reactions.** Typical of the hydrogenation catalyzed by molds are the reduction of the $\Delta^4$ double bond et al., 1952); the $\Delta^{16}$ double bond (Meister et al., 1953*a*); the s ketone (Peterson et al., 1955; Shull and Bloom, 1956; Szpilfo 1956; Agnello et al., 1955; Bloom et al., 1956); and the side-ch hyde group (Meister et al., 1954). These reactions are illustrat

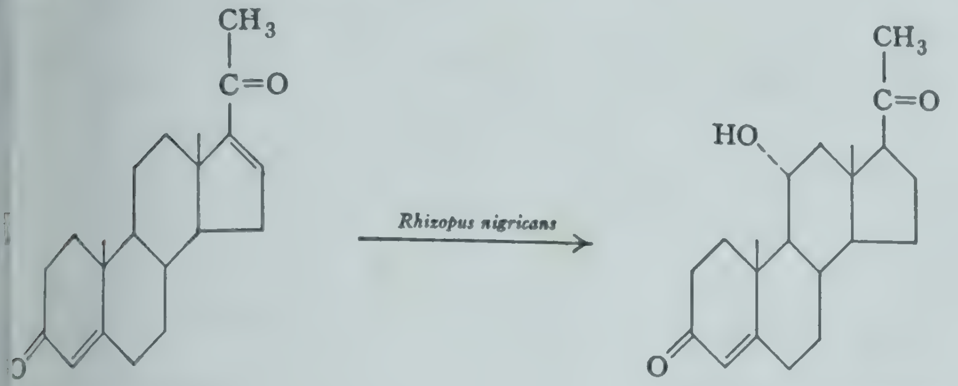
*Reduction of the $\Delta^4$ Double Bond.* A number of different scientists have shown that the $\Delta^4$ double bond of steroids may b by microorganisms. Mamoli and Vercellone (1937) showed t was able to reduce this bond. Perlman et al. (1952) repo 16α-hydroxypregnane-3,20-dione was formed concomitantly w progesterone from progesterone by an actinomycete. Peters (1952) demonstrated that 11α-hydroxyallopregnane-3,20-di formed in small amounts (0.5 to 4 per cent) by *R. nigricans* ( 6227b) from progesterone, along with 80 to 90 per cent yield hydroxyprogesterone.



Progesterone

11α-Hydroxyallopregn
3, 20-dione

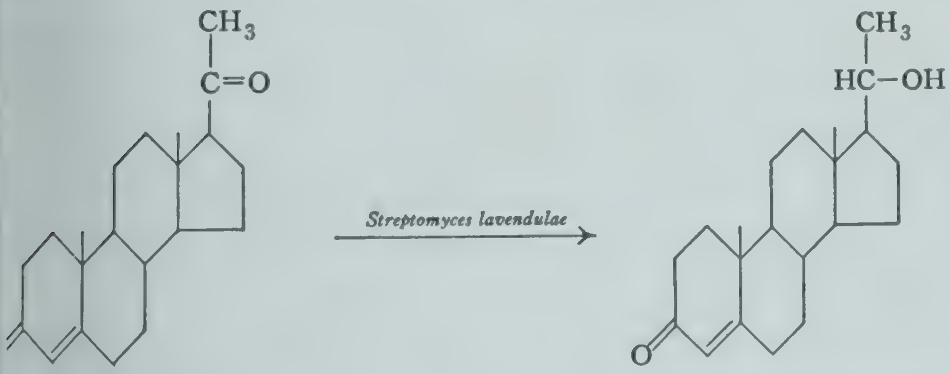
*Reduction of the* $\Delta^{16}$ *Double Bond.* Meister and associates (1953*a*) wed that *R. nigricans* (A.T.C.C. 6227b) possessed the ability to reduce $\Delta^{16}$ double bond of 16-dehydroprogesterone with the formation of -hydroxy-17α-progesterone.



16-Dehydroprogesterone

11α-Hydroxy-17α-progesterone

*eduction of Side-chain Ketone.* According to Fried et al. (1953) the etone of progesterone may be reduced in part to 20β-hydroxy-4-nene-3-one through the action of *S. lavendulae.*

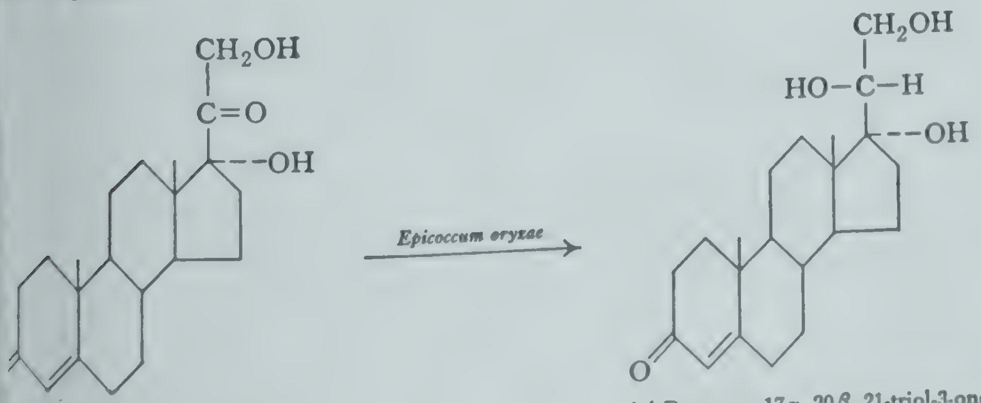


Progesterone

20β-Hydroxy-4-pregnane-3-one

erson et al. (1955) showed that the 20-ketone group of desoxy-osterone may be reduced to 20α,21-dihydroxy-4-pregnene-3-one by tinomycete.

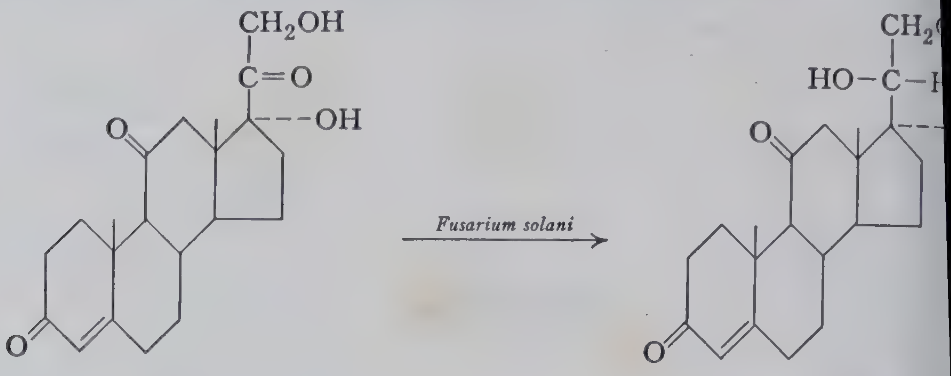
ll and Bloom (Shull, 1956) demonstrated that Reichstein's com-l S may be converted to $\Delta^4$-pregnene-17α,20β,21-triol-3-one by *Epi-m oryzae:*



Reichstein's compound S

$\Delta^4$-Pregnene-17α, 20β, 21-triol-3-one

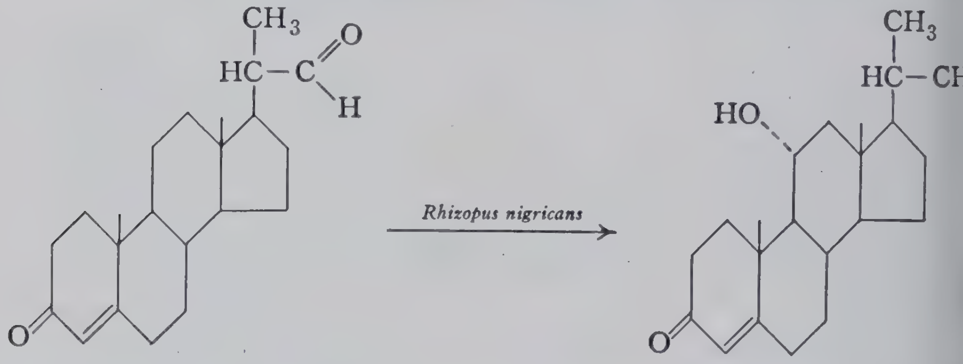
Szpilfogel and associates (1956) showed that cortisone ma verted to Reichstein's substance U by *Fusarium solani:*



Cortisone

Reichstein's substance U

*Reduction of Side-chain Aldehyde Group.* Meister et al. (1954 that the aldehyde group of 3-ketobisnor-4-cholene-22-al is reduc primary alcohol group and an 11α-hydroxyl group is introduced the action of *R. nigricans* (A.T.C.C. 6227b), as is illustrated belo



3-Ketobisnor-4-cholene-22-al

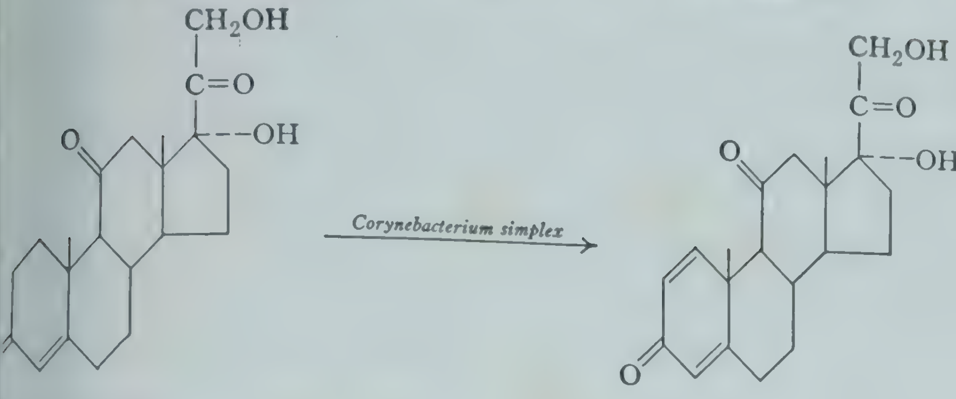
3-Ketobisnor-4-cholene-11, 22-d

Other examples of hydrogenation reactions have been report Peterson (1956) and by Shull (1956).

**Dehydrogenation Reactions.** These include the introduction 1,2 double bond into ring A of the nucleus to produce 1-dehydrost and the transformation of secondary alcohols to ketones.

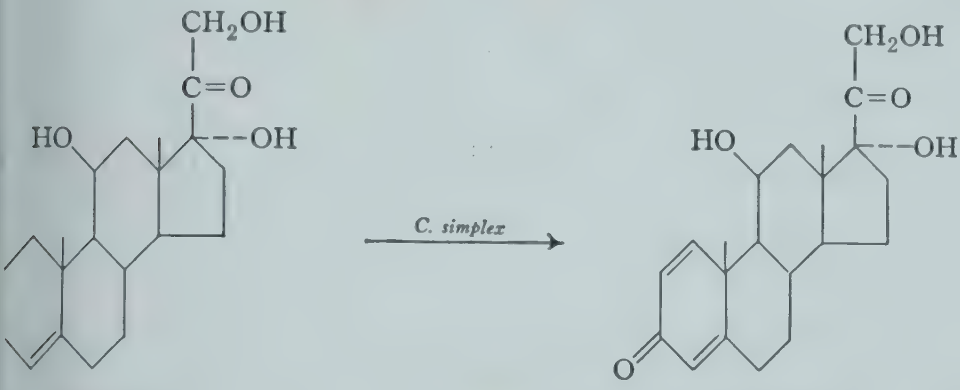
Vischer et al. (1955*a*) reported the transformation of cortis $\Delta^{1,4}$-pregnadiene-17α,21-diol-3,11,20-trione (prednisone) and Reich compound S to $\Delta^{1,4}$-pregnadiene-17α,21-diol-3,20-dione, using *F.* They converted corticosterone to $\Delta^{1,4}$-pregnadiene-11β,21-diol,3,20 and deoxycorticosterone to $\Delta^{1,4}$-pregnadiene-21-ol-3,20-dione,21-ac using *Calonectria decora.*

Nobile and coworkers (1955) showed that it was possible to pr prednisone from cortisone and $\Delta^{1,4}$-pregnadiene-11β,17α,21-triol dione (prednisolone) from cortisol through the use of *Corynebact simplex* (A.T.C.C. 6946):



Cortisone

Prednisone



Hydrocortisone

Prednisolone

procedure consisted of dissolving the cortisone (or other steroid) anol, adding the solution to shake flasks which contained a 24-hr. of *C. simplex* grown in a nutrient medium containing 0.1 per cent xtract (Difco) and buffered at pH 7, shaking the mixture for 3 r. at 28°C., extracting the transformed steroid with chloroform, ting the solvent, and crystallizing from acetone.

regnadiene-17$\alpha$,21-diol-3,20-dione; $\Delta^{1,4}$-pregnadiene-11$\beta$,21-diol-ne; and $\Delta^{1,4}$-pregnadiene-21-ol-3,20-dione, 21-acetate were pre- a similar manner, by use of *C. simplex*.

1-dehydrosteroids, prednisone and prednisolone, formed from e and hydrocortisone, respectively, are more potent in the treat- rheumatoid arthritis than are the natural corticosteroids.

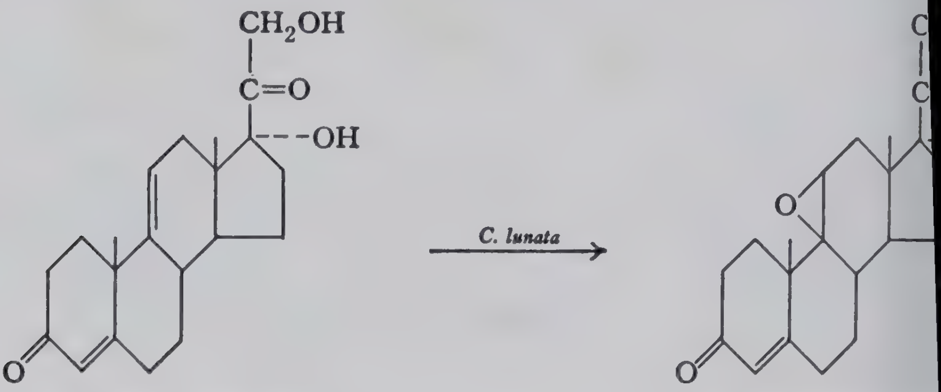
er et al. (1955*b*) found that certain molds of the genus *Didymella*, *D. lycopersici* Kleb., were able to introduce a 1,2 double bond -3-keto-steroids. They produced 1,dehydro-17$\alpha$-oxycorticosterone $\alpha$-oxycorticosterone; 1,dehydro-17$\alpha$-methyltestosterone from 17$\alpha$- stosterone; and other new 1-dehydrosteroids.

molds cleave the side chain while removing hydrogen from the 17-deoxysteroids. This is an undesirable reaction. Consequently ntial to use microbes which are known to bring about the required n without complicating reactions. Vischer and associates found that $\Delta^1$ analogs could be produced from 17-desoxysteroids ntion of the side chain, by using selected species of *Alternaria*, *ria*, and *Ophiobolus*. Later (1955*b*) they reported that *D.*

*lycopersici* produced high yields of $\Delta^1$ analogs from a variety

**Epoxidation.** Epoxidation of the isolated double bond of
rated steroid apparently takes place when it is incubated wit
organism which is capable of introducing an axial hydroxyl
the nucleus at the point of unsaturation (Shull, 1956).

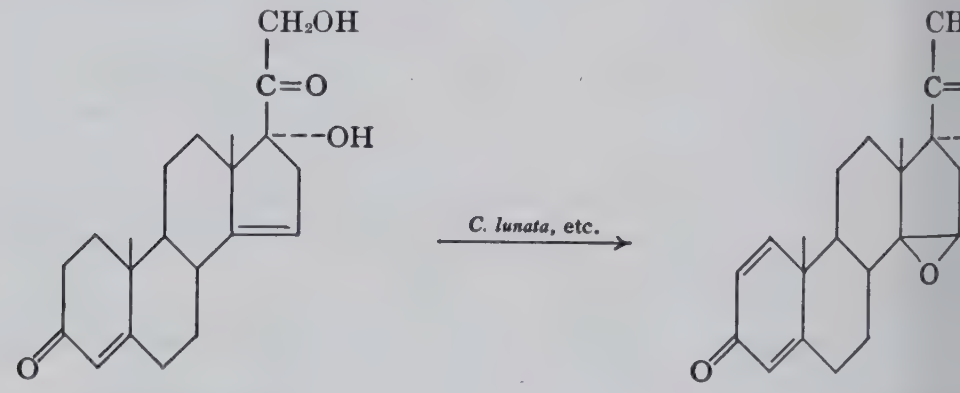
Bloom and Shull (1955) and Bloom and associates (Shull, 1
published examples of epoxidation. In one case, $\Delta^{9(11)}$-deh
pound S was transformed to 9β,11β-epoxido-compound S th
action of either *C. lunata* or *Cunninghamella blakesleeana:*



$\Delta^{9(11)}$-Dehydro-compound S

9β, 11β-Epoxido-compo

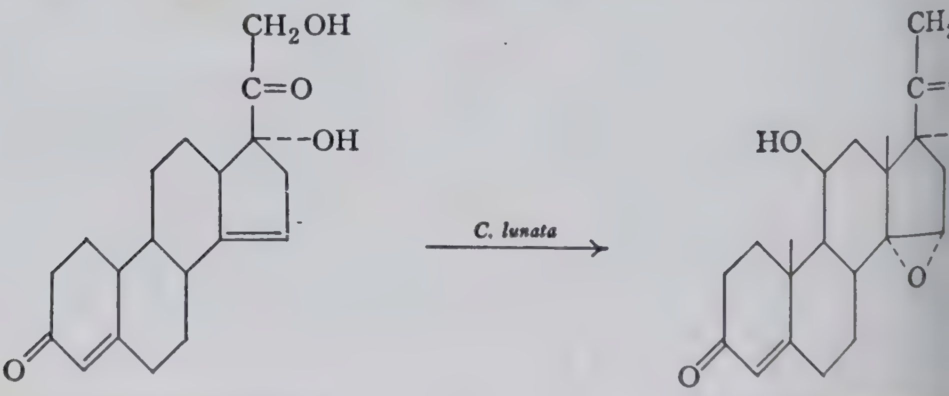
In a second case, $\Delta^{14}$-dehydro-compound S was converted into
epoxido-compound S by microbes capable of 14α-hydroxylation
*C. lunata, C. blakesleeana, Helicostylum piriforme, Mucor grise*
and *M. parasiticus:*



$\Delta^{14}$-Dehydro-compound S

14α, 15α-Epoxido-compou

In a third case, 14α,15α-epoxidohydrocortisone was obtaine
$\Delta^{14}$-dehydro-compound S through the use of *C. lunata* (Bloom et



$\Delta^{14}$-Dehydro-compound S

14α, 15α-Epoxido-hydrocor

**Reactions Involving Cleavage of the Side Chain.** Turfitt (1948) owed that *Proactinomyces* was capable of degrading the side chain of cholestenone to 3-keto-4-etiocholenic acid.

Peterson and associates (1953) reported on the degradation of $C_{21}$ roids to $C_{19}$ ketones and to testololactone. Progesterone, Reichstein's mpound S, and desoxycorticosterone were converted to 4-androstene- 7-dione by *Gliocladium catenulatum*, *P. lilacinum*, and *A. flavus*. ogesterone was degraded to 6β-hydroxy-4-androstene-3,17-dione by *catenulatum*.

Peterson and his coworkers also demonstrated that such compounds as gesterone, 17α-hydroxyprogesterone, Reichstein's compound S, and oxycorticosterone may be bioconverted to testololactone through the ion of *A. flavus*, *P. adametzi*, and other cultures of these genera.

leavage of the side chain by *Fusarium* species has been reported by cher and Wettstein (1953), and by *S. lavendulae*, by Fried and asso- es (1953). During the reaction, progesterone and desoxycorticosterone be converted to 1,4-androstadiene-3,17-dione. Simultaneously with cleavage, ring A is dehydrogenated. Peterson (1955) noted that 1,4- rostadiene-3,17-dione may be readily converted to estrone by pyroly- Estrone is an estrogenic hormone.

rogesterone, Reichstein's compound S, and testosterone may be aded to 1-dehydrotestololactone through the action of *Cylindrocarpon icola*, according to Fried et al. (1953). This conversion represents age of the side chain and ring D and also dehydrogenation of ring A. ustrations of the foregoing types of changes with progesterone will und in Fig. 116.

**eparation of Some Cortical and Gonadal Hormones.** Peterson ) suggested some methods for producing selected hormones and their atives.

gesterone and 11-deoxycortisol (Reichstein's compound S) are ble substrates for the preparation of some of the cortical and gonadal nes. 11-Deoxycortisol may be prepared from progesterone in a er of ways by use of the microbiological method. Peterson (1956) ted two alternate routes for its production. In one route, pro- one may be converted in yields of 60 per cent or greater to 11-deoxy- osterone, by use of *O. herpotrichus* (Meystre et al., 1954). The latter und may then be transformed in 30 per cent yield to 11-deoxy- ol with *C. roseum*. However, 6β,21-dihydroxy-4-pregnene-3,20-dione duced as an undesirable side product. In the second route, 17α- xyprogesterone may be converted to 11-deoxycortisol by *O. herpo- s* (Meystre et al., 1954).

isone may be prepared by a combination of microbiological and al steps (Peterson, 1956). 11-Deoxycortisol may be converted to ortisol in a yield of 70 per cent by incubation with *R. nigricans* on et al., 1953) or with *A. niger* (Fried et al., 1952). The 21-acetate latter compound may be oxidized to cortisone acetate with chro- rioxide in a yield of 80 per cent.

sol (hydrocortisone) may be obtained in 60 to 70 per cent yield by incubating 11-deoxycortisol with *C. blakesleeana* (Mann et or in 40 per cent yield by incubating the same compound wit (Shull and Kita, 1955).

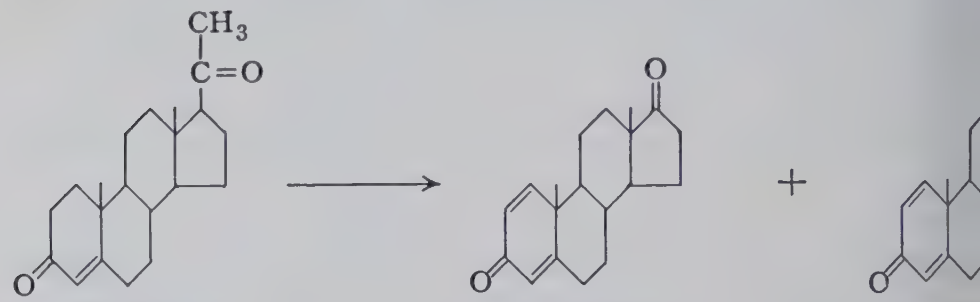
Corticosterone may be prepared from 11-deoxycorticosteron of 45 per cent with *C. blakesleeana* (Mann et al., 1955) and in 28 per cent with *C. lunata* (Shull and Kita, 1955).

11-Dehydrocorticosterone may be prepared by converting p to 11-hydroxyprogesterone with *R. nigricans* (Peterson an 1952), by oxidizing the latter compound to 11-ketoprogeste chromic acid, and by transforming the 11-keto-compound with *trichus* (Meystre et al., 1954). A yield of about 50 per cent i in the last step, whereas each of the first two steps produce quantitative yield (Peterson, 1956).

Androstenedione (refer to Fig. 114) may be prepared from pr (or a number of other $C_{21}$-compounds) through the use of select of the genera *Aspergillus, Penicillium,* and *Gliocladium* (Peter 1953*c*).

Testosterone may be prepared from androstenedione by use (Koester et al., 1941).

Androstadienedione and 1-dehydrotestosterone may be obtaine yields by incubating *F. solani* with progesterone (Peterson, 19 low yields (7 and 12 per cent, respectively) by incubating *S. la* with progesterone (Fried et al., 1953):



**Progesterone** **Androstadienedione** **1-Dehydrot**

For further details regarding the preparation of steroids, the referred to the basic papers cited in the references.

| Microorganisms | Hydroxylation |  |  |  |  |  |  |  |  |  |  |  |  | Hydrogenation |  |  |  | Dehydrogenation | Epoxidation | Cleavage |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 6β | 7α | 7β | 8 or 9 | 10 | 11α | 11β | 14α | 15α | 15β | 16α | 17α | 21 | $\Delta^4$ | $\Delta^{16}$ | Ketone | Aldehyde |  |  |  |
| Actinomycete |  |  |  |  |  |  |  |  |  |  |  |  |  | √ |  | √ |  |  |  |  |
| *Alternaria* sp. |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | √ |  |  |
| *Aspergillus amstelodami* |  |  |  |  |  | √ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| *A. chevalieri* | √ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| *A. flaviceps* |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | √ |
| *A. flavipes* |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | √ |
| *A. flavus* |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | √ |
| *A. nidulans* | √ |  |  |  |  | √ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| *A. niger* | √ |  |  |  |  | √ |  |  |  |  |  |  | √ |  |  |  |  |  |  |  |
| *A. ochraceus* |  |  |  |  |  | √ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| *A. pseudoglaucus* |  |  |  |  |  | √ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| *A. repens* |  |  |  |  |  | √ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| *Aspergillus* sp. |  |  |  |  |  | √ |  |  |  |  |  | √ |  |  |  |  |  |  |  | √ |
| *Bacillus cereus* |  |  |  |  |  |  |  | √ |  |  |  |  |  |  |  |  |  |  |  |  |
| *B. putrificus* |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | √ |  |  |  |  |

TABLE 215. SOME MICROORGANISMS CAPABLE OF TRANSFORMING STEROIDS—(*Continued*)

| Microorganisms | Hydroxylation | | | | | | | | | | | | | Hydrogenation | | | | Dehydrogenation | Epoxidation | Cleavage |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6β | 7α | 7β | 8 or 9 | 10 | 11α | 11β | 14α | 15α | 15β | 16α | 17α | 21 | $\Delta^4$ | $\Delta^{16}$ | Ketone | Aldehyde | | | |
| *B. sphaericus* | | | | | | | | | | | | | | | | | | ✓ | | |
| *Calonectria decora* | | | | | | | | | | | | | | | | | | ✓ | | |
| *Cephalothecium roseum* | | | | | | ✓ | | | | | | ✓ | | | | | | | | |
| *C. subverticillatum* | | | | | | | | | | | | | | | | | | | | ✓ |
| *Colletotrichum antirrhini* | | | | | | | | | ✓ | | | | | | | | | | | |
| *Coniothyrium* sp. | | | | | | | ✓ | | | | | | | | | | | | | |
| *Corynebacterium simplex* | | | | | | | | | | | | | | | | | | ✓ | | |
| *Cunninghamella blakesleeana* | | | | | | | ✓ | ✓ | | | | | | | | | | | ✓ | |
| *Curvularia lunata* | | ✓ | | | | | ✓ | ✓ | | | | | ✓ | | | | | | ✓ | ✓ |
| *C. pallescens* | | ✓ | | ✓ | | | | | | | | | | | | | | | | |
| *Curvularia* sp. | | | | | | | ✓ | ✓ | | | | | | | | | | | | |
| *Cylindrocarpon radicicola* | | | | | | | | | | | | | | | | | | | | ✓ |
| *Dactylium dendroides* | | | | | | ✓ | | | | | | ✓ | | | | | | | | |

| Microorganisms | Hydroxylation | | | | | | | | | | | | | Hydrogenation | | | | Dehydrogenation | Epoxidation | Cleavage |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $6\beta$ | $7\alpha$ | $7\beta$ | 8 or 9 | 10 | $11\alpha$ | $11\beta$ | $14\alpha$ | $15\alpha$ | $15\beta$ | $16\alpha$ | $17\alpha$ | 21 | $\Delta^4$ | $\Delta^{16}$ | Ketone | Aldehyde | | | |
| *Dothichiza* sp. | | | | | | | ✓ | | | | | | | | | | | | | |
| *Epicoccum oryzae* | | | | | | | | | | | | | | | | ✓ | | | | |
| *Eroteum chevalieri* | | | | | | ✓ | | | | | | | | | | | | | | |
| *Fusarium solani* | | | | | | | | | | | | | | | | ✓ | | ✓ | | ✓ |
| *Fusarium* sp. | | | | | | | | | | | | | | | | | | | | ✓ |
| *Gibberella baccata* | | | | | | | | | ✓ | | | | | | | | | | | |
| *Gliocladium catenulatum* | | | | | | | | | | | | | | | | | | | | ✓ |
| *Helicostylum piriforme* | ✓ | | | ✓ | | | | ✓ | | | | | | | | | | | ✓ | |
| *Hormodendrum viride* | | | | | | | | | ✓ | | | | | | | | | | | |
| *Lenzites abientina* | | | | | | | | | | ✓ | | | | | | | | | | |
| *Mucor corymbifer* | ✓ | | | | | | | ✓ | | | | | | | | | | | | |
| *M. griseocyanus* | | | | | | | | ✓ | | | | | | | | | | | ✓ | |
| *M. mucedo* | | | | | | | | | | | | | | | | | | | | ✓ |
| *M. parasiticus* | | | | ✓ | | | | | | | | | | | | | | | ✓ | |
| *Mucor* sp. | | | | | | | | ✓ | | | | | | | | | | | | |

TABLE 215. SOME MICROORGANISMS CAPABLE OF TRANSFORMING STEROIDS—(*Continued*)

| Microorganisms | Hydroxylation | | | | | | | | | | | | | Hydrogenation | | | | Dehydrogenation | Epoxidation | Cleavage |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6β | 7α | 7β | 8 or 9 | 10 | 11α | 11β | 14α | 15α | 15β | 16α | 17α | 21 | $\Delta^4$ | $\Delta^{16}$ | Ketone | Aldehyde | | | |
| *Neurospora crassa* | | | | ✓ | | | | | | | | | | | | | | | | |
| *Ophiobolus herpotrichus* | | | | | | | | | | | | | ✓ | | | | | ✓ | | |
| *Penicillium chrysogenum* | | | | | | | | | | | | | | | | | | | | ✓ |
| *P. lilacinum* | | | | | | ✓ | | | | | | | | | | | | | | ✓ |
| *Penicillium* sp. | | | | | | ✓ | | | | | | | | | | | | | | ✓ |
| *Pestalotia foedans* | | | | | | ✓ | | | | | | | | | | | | | | |
| *P. funera* | | | | | | | | | | | ✓ | | | | | | | | | |
| *Peziza* sp. | | ✓ | | | | | | | | | | | | | | | | | | |
| *Phycomyces blakesleeanus* | | | | | | | | | | ✓ | | | | | | | | | | |
| *Proactinomyces erythropolis* | | | | | | | | | | | | | | | | | | ✓ | | |
| *P. roseum** | | | | | | | | | | | | | | | | | | | | |
| *Pseudomonas* sp. | | | | | | | | | | | | | | | | | | ✓ | | |
| *Pycnodothis* sp. | | | | | | | | | | | | | | | | | | | | ✓ |
| *Pycnosporium* sp. | | | | | | | ✓ | | | | | | | | | | | | | |

| Microorganisms | Hydroxylation | | | | | | | | | | | | | Hydrogenation | | | | Dehydrogenation | Epoxidation | Cleavage |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6β | 7α | 7β | 8 or 9 | 10 | 11α | 11β | 14α | 15α | 15β | 16α | 17α | 21 | $\Delta^4$ | $\Delta^{16}$ | Ketone | Aldehyde | | | |
| *R. cambojae* | √ | | | | | | | | | | | | | | | | | | | |
| *R. nigricans* | √ | | | | √ | √ | | | | | | | | √ | √ | | √ | | | |
| *R. reflexus* | √ | | | | | | | | | | | | | | | | | | | |
| *Rhizopus* sp. | | | √ | | | √ | | | | | | | | | | | | | | |
| *Rhodoseptoria* sp. | | | | | | | √ | | | | | | | | | | | | | |
| *Spicaria* sp. | | | | | | | | | | √ | | | | | | | | | | |
| *Streptomyces aureofaciens* | | | | √ | | | | | | | | | | | | | | | | |
| *S. fradiae* | √ | | | | | | | | | | | | | | | | | | | |
| *S. lavendulae* | | | | | | | | | | | | | | | | √ | | | | √ |
| *Trichoderma viride* | | | | | | | | | | | | √ | | | | | | | | |
| *Wojnowicia graminis* | | | | | | | | | | | | | √ | | | | | | | |

## Some References concerning Steroids

Agnello, E. J., B. L. Bloom, and G. D. Laubach: A New Class of Biological Corticoids, *Jour. Am. Chem. Soc.,* **77**: 4684–4685 (1955).

Arnaudi, C.: Oxydations microbiennes des steroids, *Experientia,* **7**: 81–89 (

———: Researches on the Microbiological Degradation of Sterols, *Appl. M* **2**: 274–281 (1954).

Beesch, S. C., and G. M. Shull: Fermentation, *Ind. Eng. Chem.,* **48**: 1585 **49**: 1491 (1957).

Bernstein, S., L. I. Feldman, W. S. Allen, R. H. Blank, and C. E. Linden: *C & Industry,* 1956, p. 111.

Bloom, B. M., and G. M. Shull: Epoxidation of Unsaturated Steroids by organisms, *Jour. Am. Chem. Soc.,* **77**: 5767–5768 (1955).

———, ———, W. Boegemann, and G. D. Laubach: [Shull, G. M., Tran tion of Steroids by Molds, *Trans. N.Y. Acad. Sci., Ser. II,* **19**(2): (1956)].

Burton, R. B., A. Zaffaroni, and E. H. Keutmann: Paper Chromatography of S II. Corticosteroids and Related Compounds, *Jour. Biol. Chem.,* **188**: (1951).

Camerino, B., C. G. Alberti, A. Vercellone, and F. Ammannati: Diidrossi microbiologica del progesterone, *Gazz. chim. ital.,* **84**: 301–311 (1954).

Colingsworth, D. R., M. P. Brunner, and W. J. Haines: A Partial Microbic Synthesis of Adrenal Cortex Hormones, *Jour. Am. Chem. Soc.,* **74**: 238 (1952).

———, J. N. Karnematt, F. R. Hansen, M. A. Brunner, K. M. Mann, and Haines: A Partial Microbiological Synthesis of Hydrocortisone, *Jour. Chem.,* **203**: 807–813 (1953).

Dodson, R. M., A. H. Goldkamp, and R. D. Muir: Microbiological Hydr tion of $C_{19}$-Steroids at Positions C-1 and C-2, *Jour. Am. Chem. Soc.,* **79** (1957).

Dorfman, R. I., and R. A. Shipley: "Androgens—Biochemistry, Physiology Chemical Significance," John Wiley & Sons, Inc., New York, 1956.

Dulaney, E. L., W. J. McAleer, H. R. Barkemeyer, and C. Hlavac: Steroid formations by *Dactylium dendroides* (Bulliard) Fries, *Appl. Microbiol.,* **3** 374 (1955*a*).

———, ———, M. Koslowski, E. O. Stapley, and J. Jaglom: Hydroxylation of gesterone and 11-Desoxy-17-hydroxycorticosterone by *Aspergillus* and *P lium, Appl. Microbiol.,* **3**: 336–340 (1955*b*).

———, E. O. Stapley, and C. Hlavac: Hydroxylation of Steroids, Princ Progesterone by a Strain of *Aspergillus ochraceus, Mycologia,* **47**: 46 (1955).

Eppstein, S. H., P. D. Meister, H. C. Murray, and D. H. Peterson: Microbiol Transformations of Steroids and Their Applications to the Synthesis of mones, *Vitamins and Hormones,* **14**: 359–432 (1956).

———, ———, D. H. Peterson, H. C. Murray, H. M. Leigh, D. A. Lyttle, Reineke, and A. Weintraub: Microbiological Transformations of Steroids Preparation of 11-Epicorticosterone and 6β-Hydroxy-11-desoxycorticost *Jour. Am. Chem. Soc.,* **75**: 408–412 (1953*a*).

———, D. H. Peterson, H. M. Leigh, H. C. Murray, A. Weintraub, L. M. Rei and P. D. Meister: Microbiological Transformations of Steroids. VII. Pre tion of 11β-Hydroxypregnane,3,20-dione and 11α-Hydroxyallopregnane dione, *Jour. Am. Chem. Soc.,* **75**: 421–422 (1953*b*).

Finch, C. A.: *Mfg. Chemist,* **25**: 247 (1954).

Florey, K.: *Chimia,* **8**: 81 (1954).

Fried, J., R. W. Thoma, J. R. Gerke, J. E. Herz, M. N. Donin, and D. Perlman: dation of Steroids by Microorganisms. II. Hydroxylation in Position 11 Synthesis of Cortisone from Reichstein's Compound S, *Jour. Am. Chem.* **74**: 3962–3963 (1952).

——, and A. Klingsberg: III. Side Chain Degradation. Ring D Cleavage
. Dehydrogenation in Ring A, *Jour. Am. Chem. Soc.*, **75**: 5764-5765 (1953).

——, D. Perlman, J. E. Herz, and A. Borman: The Use of Microorganisms
he Synthesis of Steroid Hormones and Hormone Analogues, *Recent Progr.*
*mone Research*, **11**: 149–181 (1955).

W. J., and D. R. Colingsworth: Steroid Oxidation, U.S. Patent 2,649,401,
. 18, 1953.

and N. A. Drake: Fluorescence Scanner for Evaluation of Papergrams of
tical Hormones, *Federation Proc.*, **9**: 180 (1950).

)., and E. Riedl-Tůmová: *Pharmazie*, **9**: 877 (1954).

F. R., K. M. Mann, E. D. Nielson, H. V. Anderson, M. P. Brunner, J. N.
nematt, D. R. Colingsworth, and W. J. Haines: Microbiological Transforma-
of Steroids. VIII. Preparation of 17α-Hydroxycorticosterone, *Jour. Am.*
*m. Soc.*, **75**: 5369–5370 (1953).

P. S., E. C. Kendall, C. H. Slocumb, and H. F. Polley: The Effect of a
mone of the Adrenal Cortex (17α-Hydroxy-11-dehydrocorticosterone, Com-
d E) and of Pituitary Adrenocorticotropic Hormone on Rheumatoid Arthri-
*Proc. Staff Meetings Mayo Clinic*, **24**: 181–197 (1949).

F. W., Ch. Meystre, R. Neher, E. Vischer, und A. Wettstein: Biologische
roxylierungen von Steroiden, *Experientia*, **8(11)**: 422 (1952).

E. O., and D. N. Petsiavus: Effect of Physical Variables on Microbiological
sformation of Steroids, *Ind. Eng. Chem.*, **48**: 2213–2217 (1956).

H., L. Mamoli, and A. Vercellone: Biochemical Manufacture of Keto-
ids, U.S. Patent 2,236,574, Apr. 1, 1941.

A., and J. Horváth: Microbiological Oxidation of Sterols, *Nature*, **163**: 219
9).

. H., B. J. Magerlein, A. V. McIntosh, A. R. Hanze, G. S. Fonken, J. L.
mpson, A. M. Searcy, M. A. Scheri, and E. S. Gutsell: Chemical Studies
11-Oxygenated Steroids. I. 17α-Hydroxycorticosterone, *Jour. Am. Chem.*
**75**: 502–503 (1953).

. W. J., and E. L. Dulaney: Transformation of 11-Deoxycorticosterone by
*owicia graminis* (Mc. Alp.) Sacc. et Sacc., *Arch Biochem. and Biophys.*,
09–110 (1956*a*).

d ——: Transformation of Progesterone to 17α-Hydroxyprogesterone by
*oderma viride* Pers. ex Fries, *Arch. Biochem. and Biophys.*, **62**: 111–112
*b*).

L., und A. Vercellone: Biochemische Umwandlung von $\Delta^5$-Androstendion
androstandiol und $\Delta^4$-Testosteron Weiterer Beitrag zur Genese der Keim-
nhormone, *Ber.*, **70**: 2079–2082 (1937).

O., A. Zaffaroni, B. A. Rubin, F. Sondheimer, G. Rosenkranz, and C.
ssi: Steroids. XXXVII. A Ten Step Conversion of Progesterone to Corti-
*Jour. Am. Chem. Soc.*, **74**: 3711 (1952).

. M., F. R. Hanson, P. W. O'Connell: Effect of Enzyme Inhibitors on
biological Oxidation of Steroids, *Federation Proc.*, **14**: 251 (1955).

. D., S. H. Eppstein, D. H. Peterson, H. C. Murray, H. M. Leigh, A. Wein-
. and L. M. Reineke: Microbiological Transformations of Steroids: Intro-
on of the 14α-Hydroxy Group by Fungi of the Order *Mucorales*, *Abstr.*
*Meeting Am. Chem. Soc.*, Los Angeles, Mar. 15-19, 1953*a*, p. 5c.

——, H. C. Murray, S. H. Eppstein, L. M. Reineke, A. Weintraub, and
. Leigh: II. The Preparation of 11α-Hydroxy-17α-progesterone, *Jour. Am.*
*. Soc.*, **75**: 55–57 (1953*b*).

——, ——, G. B. Spero, S. H. Eppstein, A. Weintraub, L. M. Reineke, and
. Leigh: V. The Oxygenation of 17α-Hydroxyprogesterone to 6β,17α-Dihy-
progesterone and 11α,17α-Dihydroxyprogesterone, *Jour. Am. Chem. Soc.*,
16–418 (1953*c*).

H. Peterson, S. H. Eppstein, H. C. Murray, L. M. Reineke, A. Weintraub,
I. M. Leigh Osborn: XI. The Transformation of 3-Ketobisnor-4-cholen-
to 11α,22-Dihydroxy-bisnor-4-cholen-3-one and 6β,11α,22-Trihydroxybis-
cholen-3-one by *Rhizopus*, *Jour. Am. Chem. Soc.*, **76**: 5679-5682 (1954).

———, L. M. Reineke, R. C. Meeks, H. C. Murray, S. H. Eppstein, H. M. Osborn, A. Weintraub, and D. H. Peterson: XII. 17 *alpha*-Hydroxylation *Am. Chem. Soc.,* **76**: 4050 (1954*b*).

Meystre, Ch., E. Vischer, und A. Wettstein: Mikrobiologische Hydroxylierun Steroiden in der 17$\alpha$- und 21-Stellung, *Helv. Chim. Acta,* **37** (fasc. 5): 154 (1954).

———, ———, und ———: Mikrobiologische Hydroxylierungen von Cortexon i 7$\alpha$-, 15$\alpha$-oder 15$\beta$-Stellung, *Helv. Chim. Acta,* **38** (fasc. 2): 381–393 (1955).

Murray, H. C., and D. H. Peterson: Oxygenation of Steroids by *Mucorales* F U.S. Patent 2,602,769, July 8, 1952.

——— and ———: Steroids, U.S. Patent 2,649,400, Aug. 18, 1953*a*.

——— and ———: Steroids, U.S. Patent 2,649,402, Aug. 18, 1953*b*.

——— and ———: U.S. Patent 2,666,070, Jan. 12, 1954*a*.

——— and ———: U.S. Patent 2,673,866, Mar. 30, 1954*b*.

——— and ———: U.S. Patent 2,692,273, Oct. 19, 1954*c*.

——— and ———: Canadian Patent 506,689, 1954*d*.

——— and ———: Canadian Patent 508,652, Dec. 28, 1954*e*.

——— and ———: U.S. Patent 2,702,809, 1955*a*.

——— and ———: U.S. Patent 2,703,326, 1955*b*.

——— and ———: U.S. Patent 2,703,806, 1955*c*.

Nobile, A., W. Charney, P. L. Perlman, H. L. Herzog, C. C. Payne, M. E. Tu M. A. Jevnik, and E. B. Hershberg: Microbiological Transformation of Steroi I. $\Delta^{1,4}$-Diene-3-ketosteroids, *Jour. Am. Chem. Soc.,* **77**: 4184 (1955).

Pederson, R. L., J. C. Babcock, S. H. Eppstein, P. D. Meister, H. C. Murray, R. Meeks, A. Weintraub, L. M. Reineke, and D. H. Peterson: XIV. The Prepar tion of a Tertiary Hydroxy-steroid, 10$\epsilon$-Hydroxy-19-nortestosterone, *Jour. A Chem. Soc.,* **78**: 1512–1513 (1956).

Perlman, D.: Microbiological Conversion of Pregnenolone to Progesterone, *Scienc* **115**(2993): 529 (1952).

———, E. Titus, and J. Fried: The Microbiological Hydroxylation of Progesteron *Jour. Am. Chem. Soc.,* **74**: 2126 (1952).

Peterson, D. H.: Microbiological Approaches to the Preparation of Cortisone an Hydrocortisone, *Research,* **6**: 309–319 (1953).

———: Microorganisms and Steroid Transformations, Chap. 9 in "Perspectives an Horizons in Microbiology," symposium, edited by S. A. Waksman, Rutgers Uni versity Press, New Brunswick, N.J., 1955.

———: Microbiological Conversion of Steroids, *Record Chem. Progr.,* **17**(4): 211–24 (1956).

———, S. H. Eppstein, P. D. Meister, B. J. Magerlein, H. C. Murray, H. M. Leigh A. Weintraub, and L. M. Reineke: IV. The 11-Epimer of Compound F an Other New Oxygenated Derivatives of Reichstein's Compound S, a New Rout to Cortisone, *Jour. Am. Chem. Soc.,* **75**: 412–415 (1953*a*).

———, P. D. Meister, A. Weintraub, L. M. Reineke, S. H. Eppstein, H. C. Murray and H. M. Leigh Osborn: XIII. Oxygenation of 16 *alpha*, 17 *alpha* Oxidopro gesterone to 11 *alpha*-Hydroxy-16 *alpha*, 17 *alpha*-Oxidoprogesterone by *Rhizo pus nigricans* Ehrb. (ATCC 6227*b*), *Jour. Am. Chem. Soc.,* **77**: 4428–4429 (1955)

——— and H. C. Murray: Microbial Oxygenation of Steroids at Carbon-11, *Jour Am. Chem. Soc.,* **74**: 1871–1872 (1952).

———, ———, S. H. Eppstein, L. M. Reineke, A. Weintraub, P. D. Meister, and H. M. Leigh: I. Introduction of Oxygen at Carbon-11 of Progesterone, *Jou Am. Chem. Soc.,* **74**: 5933–5936 (1952).

———, A. H. Nathan, P. D. Meister, S. H. Eppstein, H. C. Murray, A. Weintraub L. M. Reineke, and H. M. Leigh: VI. Preparation of 11$\alpha$-Hydroxy-6-dehydro- progesterone, *Jour. Am. Chem. Soc.,* **75**: 419–421 (1953*b*).

Rubin, B. A., C. Casas-Campillo, G. Hendrichs, F. Cordova, and A. Zaffaroni: The 21-Hydroxylation of Progesterone and Its Analogs by Microorganisms, *Bac Proc.,* 1956, p. 33.

Shull, G. M.: Transformations of Steroids by Molds, *Trans. N.Y. Acad. Sci., Ser. II* **19**(2): 147–172 (December, 1956).

and B. M. Bloom: Microbiological Epoxidation of Unsaturated Steroids, *Congr. intern. Biochem., Brussels*, Aug. 1–6, 1955.

J. W. Davisson, and J. B. Routien: British Patent 740,848, 1955.

and D. A. Kita: Microbiological Conversion of Steroids. I. Introduction of *eta*-Hydroxyl Group into $C_{21}$ Steroids, *Jour. Am. Chem. Soc.*, **77**: 763 (1955).

——, and J. W. Davisson: Oxygenation of Steroids, U.S. Patent 2,658,023, v. 3, 1953.

——, and ——: U.S. Patent 2,702,812, 1955.

J. L. Sardinas, and J. B. Routien: Canadian Patent 507,009, Nov. 2, 1954.

A. R., and R. J. Hickey: "Industrial Fermentations," Chemical Publishing npany, Inc., New York, 1954, Vol. 2, p. 387.

)., M. Hayano, R. I. Dorfman, O. Hechter, C. R. Robinson, and C. Djerassi: droxylation of Desoxycorticosterone with *Neurospora crassa, Jour. Am. m. Soc.,* **77**: 3926–3927 (1955).

T. H., W. J. McAleer, J. M. Chemerda, M. A. Kozlowski, R. F. Hirschmann, Marlatt, and R. Miller: The Microbial 1-Dehydrogenation of Some $\Delta^4$-3-Ketooids, *Arch. Biochem. and Biophys.*, **59**: 304–305 (1955).

l, S. A., M. S. DeWinter, and W. J. Alsche: *Rec. trav. chim.*, **75**: 402 (1956).

G. E.: The Microbiological Degradation of Steroids: IV. Fission of the oid Molecule, *Biochem. Jour.*, **42**: 376–383. (1948).

E., Ch. Meystre, und A. Wettstein: Mikrobiologische Herstellung von hydro-Steroiden, *Helv. Chim. Acta*, **38** (fasc. 3): 835–840 (1955*a*).

——, und ——: Herstellung weiterer 1-Dehydro-steroide auf mikroogischem Wege, *Helv. Chim. Acta*, **38** (fasc. 6): 1502–1507 (1955*b*).

J. Schmidlin, und A. Wettstein: 16-Oxy-cortexon, *Helv. Chim. Acta*, **37** c. 1): 321–326 (1954).

und A. Wettstein: Mikrobiologische Reaktionen, *Experientia*, **9**(10): 371 3).

M., et C. Heusghem: Streptomyces transformant l'oestradiol en oestrone, *pt. rend. soc. biol.*, **142**: 1074–1076 (1948).

n, A.: Conversion of Steroids by Microorganisms, *Experientia*, **11**: 465–479 5).

d G. Anner: Advances in the Field of Adrenal Cortical Hormones, *Experi-* , **10** (fasc. 10): 397–416 (1954).

A., and R. B. Burton: Identification of Corticosteroids of Beef Adrenal ct by Paper Chromatography, *Jour. Biol. Chem.*, **193**: 749–767 (1951).

——, and E. H. Keutmann: Adrenal Cortical Hormones: Analysis by Partition Chromatography and Occurrence in the Urine of Normal Per- *Science*, **111**: 6–8 (1950).

. Casas Campillo, F. Cordova, and G. Rosenkranz: Steroids. LXVI. Microgical Hydroxylation in Position 21, *Experientia*, **11**(6): 219 (1955).

## CHAPTER 45

# ANTIBIOTICS

An "antibiotic" is a chemical substance produced by a living org that demonstrates inhibitory or germicidal activity toward micro isms in vivo or in vitro. Usually the term is applied to compound duced by microorganisms. However, some scientists believe that the should include the antimicrobial substances produced by higher pla well. The antibiotic activity is sometimes referred to as being "ant terial," "antifungal," or "antimicrobial."

Antibiotics are produced by certain fungi, bacteria, actinomycetes ally regarded as being closely related to the bacteria), algae, and lic Substances with similar properties are produced by some seed p (Florey et al., 1949; Lucas et al., 1955).

The most important and fertile source of commercially important biotics is the genus *Streptomyces* of the actinomycetes. A large pro tion of the antibiotics used in the fields of medicine and agriculture for food preservation is produced by species of *Streptomyces*. Infor tion concerning the antibiotics formed by actinomycetes has been viewed by Waksman and Lechevalier (1953), Benedict (1953), others. Background information on the actinomycetes has been prov by Skinner, Emmons, and Tsuchiya (1947); Waksman (1950, 1 1953); Perlman (1953); Gottlieb (1953); Villemin, Lechevalier, Waksman (1953); Burkholder et al. (1954); Hesseltine, Benedict, Pridham (1954); and others.

Bacteria are sources of a considerable number of antibiotics. Howe only a few of these are of commercial importance. Among the latter bacitracin, polymyxin B, tyrothricin, and nisin.

Many molds also produce antibiotics. The penicillins are the outsta ing examples of mold-produced antibiotics. Griseofulvin shows prom in the field of plant protection, but its real value has not been defini established. Synnematin $B_1$ also shows promise. Florey et al. (19 have supplied a storehouse of information on the penicillins and m data on the antibiotics formed by molds. Brian (1951) has reviewed subject of the antibiotics produced by fungi.

Table 216 shows some antibiotics of proved value or of large prom

Information relative to the kinds, numbers, and distribution of micro which produce antibiotics may be obtained by reference to the publi tions of Florey et al. (1949); Brian (1951), who surveyed the fur Raper (1952), who referred to the filamentous, saprophytic molds. bacteria, the actinomycetes, the higher fungi, and the seed plants; Be

BLE 216. Some Antibiotics of Proved Value or of Promise in the Fields of Medicine, Food Technology, and Agriculture

Actinomycin
Amphotericins
Anisomycin
Bacitracin
Candicidin
Carbomycin
Chloramphenicol
Chlortetracycline
Cycloheximide
Cycloserine
Erythromycin
Eulicin
Fumagillin
Griseofulvin
Neomycin
Nisin
Novobiocin
Nystatin
Oleandomycin
Oxytetracycline
Penicillins
Polymyxin
Puromycin
Ristocetin
Streptolydigin
Streptomycin
Subtilin
Synnematin B
Tetracycline
Thiolutin
Thiostrepton
Tyrothricin
Vancomycin
Viomycin

1953), who reported on the actinomycetes; Waksman and Leche-
(1953), who also discussed the actinomycetes; and others.

**Development of a New Antibiotic.** Table 217 lists some of the
ns which must be considered in the development of a new anti-

LE 217. Some Problems of Research, Development, and Manufacture Relating to an Antibiotic

- Isolation or collection of cultures
- Screening of cultures to detect those with antimicrobial activity
- Development of methods for submerged-culture production:
  - Development of media
  - pH adjustment and control
  - Optimum temperature
  - Optimum conditions of aeration
  - Control of foam formation
  - Optimum time or length of fermentation
  - Other factors
- Development of methods for isolation and purification:
  - Determination of properties of antibiotic:
    - Physical:
      - Solubilities in solvents
      - Stability in acids, alkalies, heat, etc.
      - Adsorption and absorption
    - Chemical:
      - Reactions
- Evaluation of antibiotic:
  - Pharmacological tests:
    - Toxicity in animals (mice, rats, guinea pigs, dogs, monkeys)
    - Therapeutic effect on experimental infections
  - Antimicrobial action (spectrum):
    - Bacteriostatic action
    - Bactericidal action
    - Fungistatic action
    - Fungicidal action

TABLE 217. SOME PROBLEMS OF RESEARCH, DEVELOPMENT, AND MANUFACT
RELATING TO AN ANTIBIOTIC—(*Continued*)

        Antiviral action
        Other
    Comparison with existing antibiotics
Development of pilot-plant-production methods
Preparation of pure product for clinical testing and evaluation:
    Determination of resistance
    Determination of synergistic action
Development of plant-scale-production methods
Testing of purified antibiotic:
    Potency
    Sterility
    Pyrogenicity
    Toxicity
    Histamine response
    Moisture
    pH
    Heat stability
    Crystallinity
Miscellaneous other considerations:
    Development of methods to control production of antibiotic
    Determination of chemical structure of antibiotic
    Determination of mode of action of antibiotic
    Development of new applications
    Development of marketing and distribution system
    Financing of business

**Factors Considered in Selection of Antibiotic.** Only a comp tively few of the many hundreds of antibiotics produced by microor isms have been selected for manufacture on an industrial scale. A biotics for use in the field of medicine should be relatively nont They should not precipitate the serum proteins, cause hemolysis of blood, or adversely affect the phagocytes. They should be solubl water, reasonably stable, and effective against pathogens under the ditions of use. They should be free of pyrogenicity and should not c histamine-like responses. They should be well tolerated by the indivi in the doses required. They should bring about as few undesirable responses as possible. Obviously no antibiotic is perfect, and even best of them occasionally cause an undesirable reaction in the pati Hussar and Holley (1953) have discussed many of the reactions antibiotics.

Antibiotics possessing rather obvious disadvantages are occasion manufactured on a commercial scale because of their outstanding va for the chemotherapy of a particular disease or condition. Others especially effective against antibiotic-resistant strains of bacteria, s as *Staphylococcus aureus*. Some may be used topically only.

## SOME ISOLATION METHODS AND SCREENING TECHNIQUE

Excellent discussions of methods for isolating and screening cultu for antibiotic production have been made by Waksman (1947). Flo

(1949), Pratt and Dufrenoy (1953), and others. Duggar (1950) ed on the isolation of culturable fungi from diverse habitats, Waks- and Lechevalier (1951) discussed the principle of screening anti- -producing organisms, and Routien and Finlay (1952) reviewed of the problems concerned in the search for microorganisms pro- antibiotics.

**ne Isolation Methods.** No attempt will be made here to review the hat voluminous literature on this subject. The interested reader is d to Waksman's book, "Microbial Antagonisms and Antagonistic nces," for a general review of this field.

procedure known as the "crowded-plate method," 1-ml. portions of o 1:1,000 dilutions of soil are plated with nutrient agar and incu- at temperature ranges suitable for the growth of bacteria. Antag- organisms (those producing antibiotic substances) will produce ones around their colonies as a result of inhibiting the growth of nt bacteria or of destroying them. Such organisms are isolated in lture, and the antibiotic is extracted, studied, and identified.

direct-soil-inoculation method, plates are poured with nutrient hich has been seeded with the organism for which an antagonist is . The plates are incubated at 28 or 37°C. for 1 to 2 days. Then s of soil (fresh or enriched) are dropped onto the surface of the on the plate, which is then incubated for a further period. An nistic organism will inhibit, destroy, or cause lysis of the original m. Potato agar may be used instead of nutrient agar when it is to isolate an organism antagonistic to fungi, according to an.

ionally the researcher is interested in isolating new strains of a ar species or of a group of organisms for the purpose of obtaining that is a better producer of a given antibiotic than known strains. Alexander, and Coghill (1944) desired to isolate new strains of the *um notatum-chrysogenum* group and proceeded in the following manner: The isolations were made by dilution and streak culture . In the former method, suitable dilutions of soil samples, usually to 1:1,000,000, were plated in Czapek's medium or in an acid nitrate agar. The plates were incubated for 5 to 10 days at 24 . and then examined for molds displaying the desired character- the penicillin-producing strains of the *P. notatum-chrysogenum* The streak method was carried out by streaking the surface of ision agar with 1 to 4 loopfuls of a 1:10 suspension of soil, incu- he plates at 24 to 26°C. for 7 to 10 days, and examining the plates he desired species. Final isolations of molds grown on the hay agar were made in Czapek's medium.

olds that appeared to be members of the *P. notatum-chrysog-* oup were next planted on the surface of Czapek's solution agar dishes by Raper and his associates, an attempt being made to two to four colonies, equally spaced, in each plate. The plates ubated at room temperature for 10 to 12 days. Then the penicil- dia, and other characteristics of each mold were examined to determine whether it possessed the desired qualifications. The ance of an abundant yellow exudate was one of the distinguish tures.

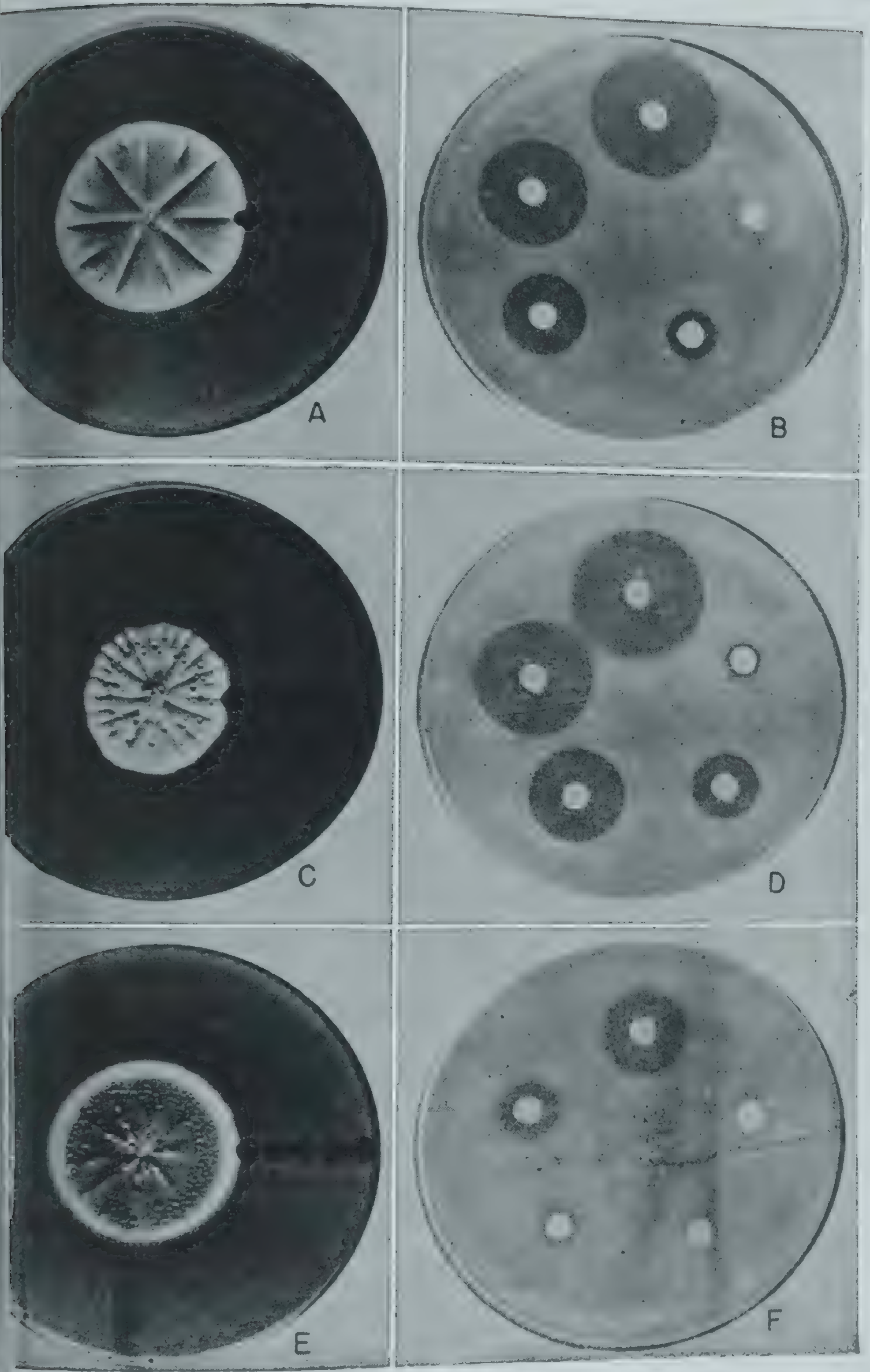
Provided that the mold turned out to be a member of the *P. n* *chrysogenum* group, it was next submitted to a screening test t some idea of its value as a penicillin producer.

Raper and his coworkers used a medium designated as "E" to grow the molds. This medium was prepared by adding 1 per volume of corn-steep liquor (55 per cent solids) to Czapek's s adjusting the pH to 7, adding 2 per cent agar, distributing measuring exactly 20 ml. in tubes, and sterilizing. The contents tube were poured into a sterile petri dish of 100-mm. diameter flat bottom, care being taken to use dishes that would provide a u depth of agar. A single colony was produced in the center of eac as a result of inoculation with a small amount of a solidified suspen the mold spores (prepared by suspending spores in melted agar at and allowing the agar to solidify). Control plates were prepared wi different cultures of *P. notatum* known to be good pencillin pro Each plate of the series was incubated for 6 days at 24°C.

At the end of the incubation period, four or five plugs were cu removed from each plate (the first one being adjacent to the mold co and placed on the surface of an agar medium seeded with *Staphyloc* *aureus* (refer to the cylinder assay method of Schmidt and Moyer, Raper and his associates used a No. 2 cork borer with an inside dia of 5.5 mm. for cutting the plugs, which were removed to the assay me by means of a flat-pointed inoculation needle and arranged as indica sections *B*, *D*, and *F* of Fig. 119. The assay plates were incubated night at 37°C. and the zones of inhibition measured to the neares mm., using a reading glass. The results obtained were compared those from the controls, and the mold strains that produced zon inhibition on the assay plates as large as or larger than the controls saved for further study.

**Screening Techniques.** The purpose of the screening technique determine antibacterial, antifungal, or antiviral action and to sep promising isolates from those of little or no value.

There are a number of methods for screening antibacterial and fungal isolates. Florey and associates (1949) described several me in detail. In general, procedures are of two types: those in whic isolate is used with several test organisms simultaneously and tho which one or more isolates, or their extracts, are used with one organism at a time. In one popular procedure, the isolate is streaked a one side of the plate and incubated at its optimum temperature un has produced antibiotic activity in the growth medium. Cultures of to seven test organisms are then streaked at right angles to the is (Fig. 120), starting the inoculation at the edge of the isolate and w ing away from it, thus assuring the presence of an adequate inoc close to the isolate. The plates are incubated at the optimum temp ture of the test organisms. The test organisms are inhibited by the



Screening test. *A*, control culture, *Penicillium notatum* NRRL 1249.B21,
ium; incubation at 24°C.; radial series of plugs cut at 6 days, ×⅔. *B*, agar
plate showing zones of inhibition of *Staphylococcus* developed after agar
oved from *A* have been incubated for 16 hr. at 37°C., ×⅔. *C*, new isolate
illin-producing strain, incubation and test as in *A*. *D*, assay plate for same.
late, poor penicillin-producing strain, incubation and test as in *A*. *F*, assay
ame. [*Courtesy of K. B. Raper, D. F. Alexander, and R. D. Coghill, Jour.*
): 639 (1944).]

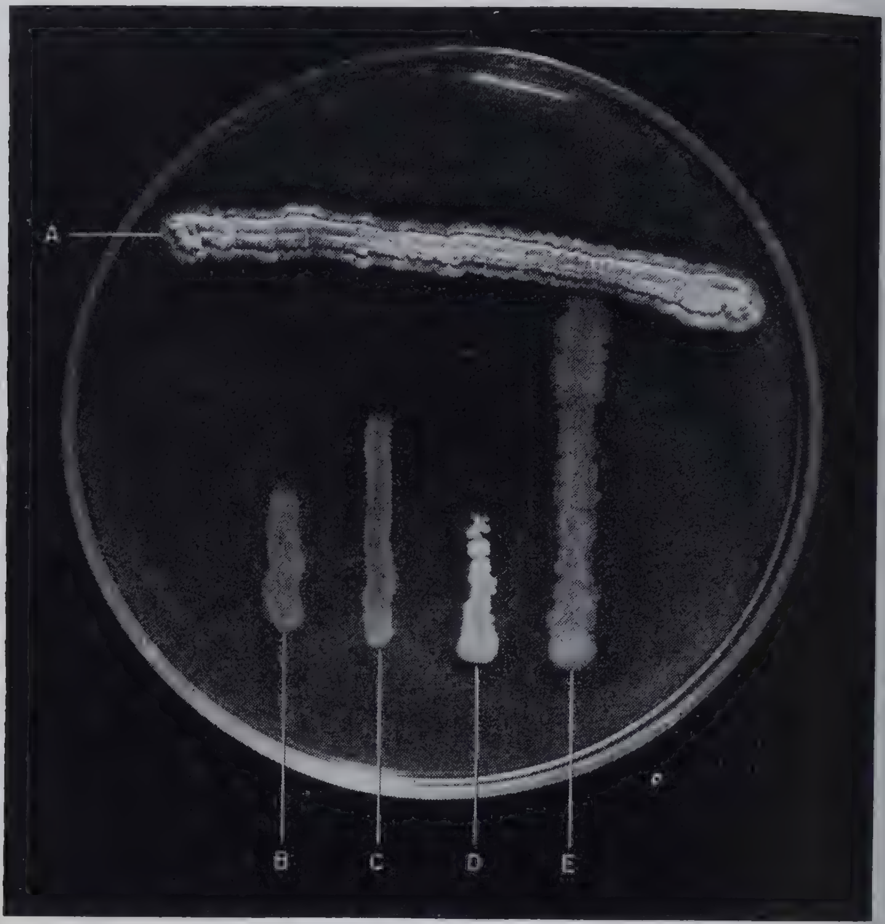


FIG. 120. Screening test. *A* is a mold and *B, C, D,* and *E* are test bacteria. (*Co of William Peters, Lederle Laboratories Division, American Cyanamid Co., York.*)

late if they are sensitive to the antibiotic substance produced by it width of the clear zone depending on the degree of sensitivity tc antibiotic. The isolate may be inoculated into the center of plate i alternate method and cultures of the test organisms streaked from edge of the developed colony to the edge of the plate like the spoke a wheel.

In some cases, as, for example, with molds, the isolate may not g well in the medium used to support the growth of the test organisms one method, the isolate may be grown in a suitable solid medium plugs cut from the fungus and the medium at different locations (refe Fig. 119) and placed on top of agar seeded with the test organism. another method, the isolate may be cultivated in a suitable liquid med to produce antibiotic activity. Portions of the crude medium, or dilut of it, may then be placed in cylinders placed on agar seeded with a organism. This procedure is a modification of the cylinder-plate as method. Paper disks may be impregnated with liquid medium from isolate and placed on the surface of a seeded medium. This metho related to the sensitivity test.

To determine whether the isolate possesses antiviral activity, i necessary to grow it in a suitable liquid medium. The microbes are se from the medium by filtration (the membrane or molecular filter work well here). The clear filtrate is used to treat chick embryos, or other animals, which have been infected with a virus. A modified que may be used with rickettsia, protozoa, or other microbes.

## TESTS TO DETERMINE IDENTITY OF ANTIBIOTIC

rder to avoid the development of antibiotics which have already tudied, it is essential to carry out tests on crude solutions of newly d antibiotics. Fischbach and Levine (1953), Grove and Randall , and others have described tests for the qualitative identification biotics. Although a number of tests are helpful for indicating the e of a given antibiotic, the obtainment of data relating to infra- d ultraviolet absorption, crystallography, melting points, and bio- is essential for confirmation of the identification. The use of paper tography is often a valuable tool for this type of testing. Other ant information may be secured by studying the antimicrobial ns of the crude substance with those of known antibiotics, by use ific strains of microorganisms, particularly resistant ones, such as *lococcus aureus.* Penicillinase may be of considerable help if the ubstance happens to be a penicillin.

## PRODUCTION METHODS

**ral Methods.** Many of the specific details regarding the plant- oduction of a given antibiotic, with certain exceptions, remain crets. However, the general methods used are a matter of com- owledge to those connected with the industry.

ntibiotic-producing microbe is cultivated in a medium and under ns which will favor growth and the production of the highest f the antibiotic. The medium will contain, in addition to the urces of carbon, nitrogen, minerals, and buffers, precursors if e known to be of value for increasing the total yield or the yield en type of antibiotic, as in the case of penicillin.

edium may be sterilized directly in the fermentor, or it may be l by a continuous or batch method and run into the previously d fermentor. It is inoculated with a suitable starter and then ned at pH and temperature levels deemed best for maximum These levels may be controlled automatically.

uate oxygenation is highly important for the successful production ntibiotic. Foaming of the mash, which is directly related to the f the medium and the degree of aeration, is controlled through of antifoam agents, which must be nontoxic to the production and must not interfere with recovery of the antibiotic.

n ingredients may be added during the fermentation to increase ls; for example, sugar may be added continuously or in incre- Precursors, when used, must be added in small amounts and at ich will not be toxic to the producing microbe.

The course of the fermentation, which generally requires sever
is followed carefully by the laboratory.

**Production of Penicillin.** There have been a relatively large
of publications on penicillin production. Among them are the re
Clutterbuck, Lovell, and Raistrick (1932); Challinor (1942); C
and MacNaughton, and Foster, Woodruff, and McDaniel (1943);
han; Coghill; Cook, Tulloch, Brown, and Brodie; Elder; Mer
Company; Pearl and Appling; Raper, Alexander, and Coghill; Sri
and Waksman and Reilly (1944); Koffler, Knight, Perlman, and
Koffler, Knight, Emerson, and Burris; Raper and Alexander; Ra
Coghill; and White, Krampitz, and Werkman (1945); Bowden and
son; Foster, Woodruff, an
Daniel; Foster, Woodruff, Pe
McDaniel, Wilker, and He
Moyer and Coghill; Raper and
nell; Stefaniak, Gailey, Brown
Johnson; and Stefaniak, Gailey
vis, and Johnson (1946); Moye
Coghill, and Taylor (1947); F
et al. (1949); Pratt and Duf
(1953); and others.

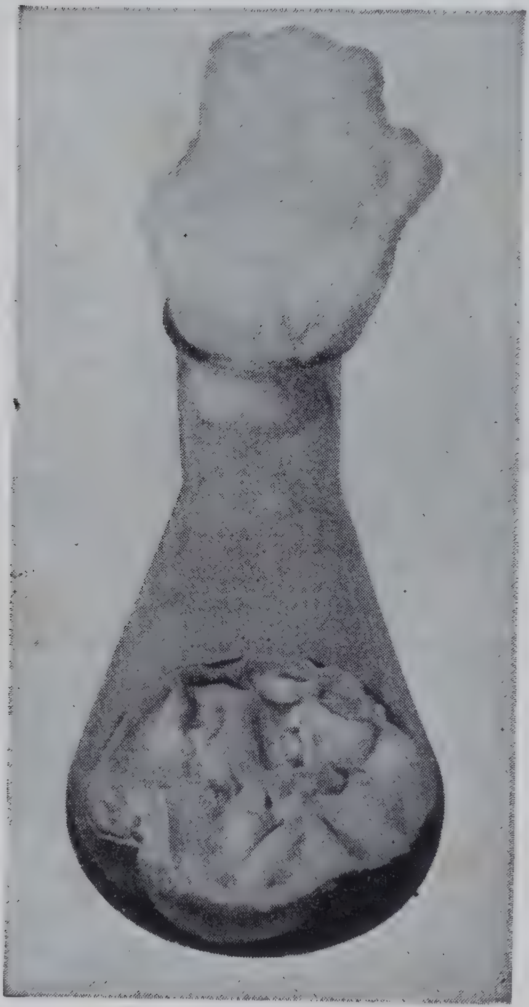
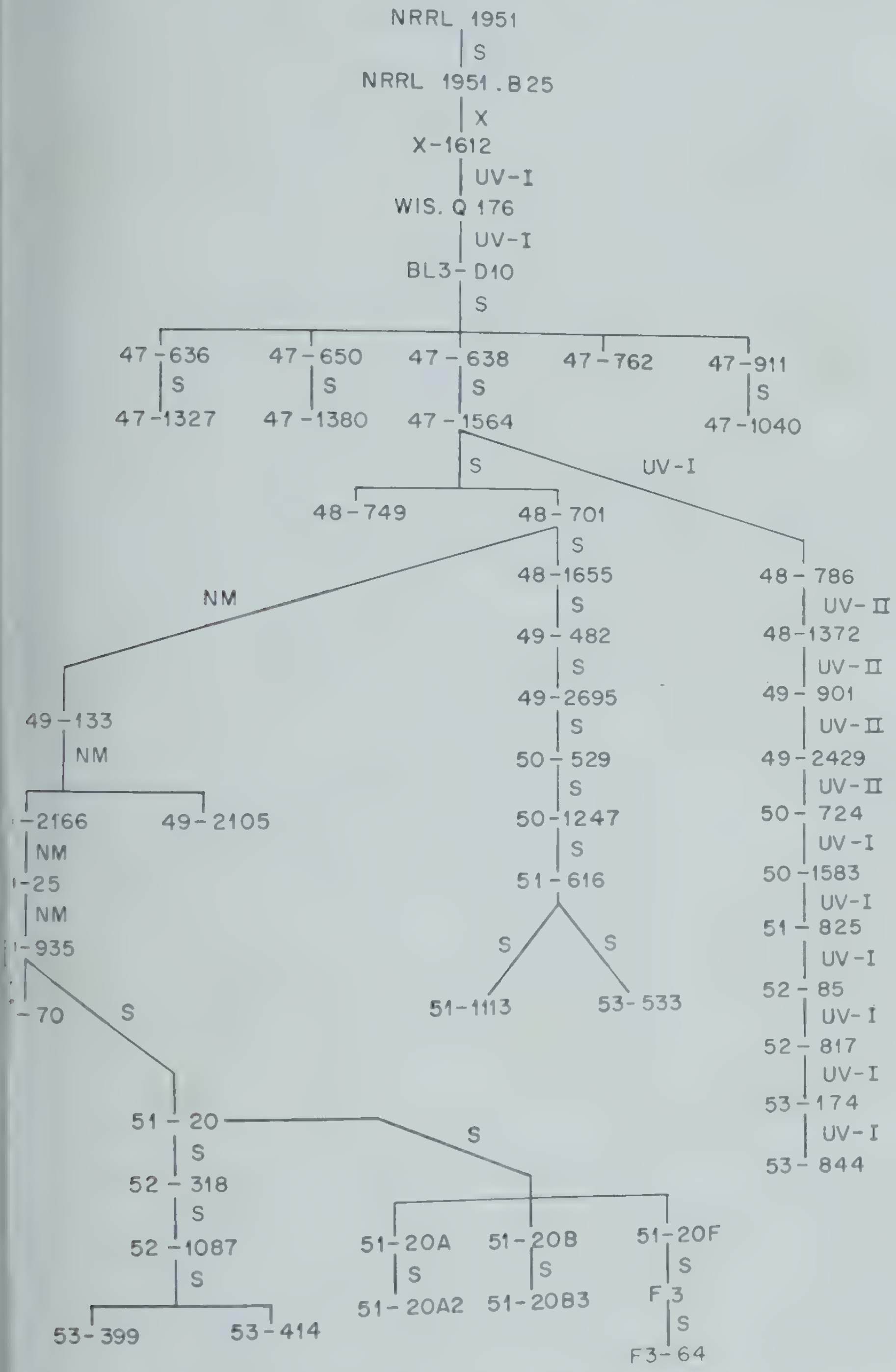


FIG. 121. Surface-culture production of penicillin by *Penicillium notatum* NRRL 1249.B21. (*Photo by Cecil G. Dunn.*)

Penicillin may be produce
both surface-culture and subme
culture methods. In the surface
ture methods, the mold is grow
the surface of shallow layers o
fermentation or production me
or of bran on a laboratory, p
plant, or commercial scale. In
submerged-culture methods, the
is grown submerged in the fern
tation medium in shake flasks, ro
drums, or deep tanks. The sh
flasks are usually used for labora
studies, while the rotary drums
deep tanks are used for pilot-p
or commercial production. Agita
and aeration of the medium are
sential in the submerged-cul
processes.

*Molds Used.* Nothing is more
portant in the production of an a
biotic than the selection of the
propriate strain. Among the significant researches in this area may
mentioned those of Raper et al. (1944); Raper and Alexander (19
Raper and Fennell (1946); Backus, Stauffer, and Johnson (1946);
Somer (1947); Anderson et al. (1953); and others.

The history of the development of improved strains through natu on, the use of irradiation methods, the use of nitrogen-mustard ent, and other procedures is most interesting. Table 218 provides ation relating to some molds used during the early years of penicil- duction. Figure 122 illustrates the derivation of some important of *Penicillium chrysogenum*, which are pigment-free and which


NRRL 1951
S
NRRL 1951.B25
X
X-1612
UV-I
WIS. Q 176
UV-I
BL3-D10
S
47-636
47-650
47-638
47-762
47-911
S
S
S
S
47-1327
47-1380
47-1564
47-1040
S
UV-I
48-749
48-701
S
48-1655
NM
48-786
S
UV-II
49-482
48-1372
S
UV-II
49-2695
49-901
49-133
S
UV-II
NM
50-529
49-2429
S
UV-II
-2166
49-2105
50-1247
50-724
NM
S
UV-I
-25
51-616
50-1583
NM
UV-I
-935
S
S
51-825
UV-I
-70
S
51-1113
53-533
52-85
UV-I
52-817
UV-I
51-20
S
53-174
S
UV-I
52-318
53-844
S
52-1087
51-20A
51-20B
51-20F
S
S
S
S
51-20A2
51-20B3
F3
S
53-399
53-414
F3-64

Genealogy of the Wisconsin family of strains of *Penicillium chrysogenum*. s selection without mutagenic treatment; *X* indicates selection following ion; *UV*-I indicates selection following 2750A *UV*-irradiation; *UV*-II indi- tion following 2534-37A *UV*-irradiation; *NM* indicates selection follow- n mustard treatment. [*Courtesy of M. P. Backus and J. F. Stauffer,* , **47**(4): 429 (1955).]

Table 218. Information concerning Some Molds Used for Penicillin Prod

| Designation of mold | History | Use |
|---|---|---|
| *Penicillium notatum*, NRRL 1249.B21 | Substrain derived from Fleming strain and developed at NRRL. More widely used than any other strain for surface-culture production in U.S. and abroad | Surface-cul productio industry |
| *P. notatum*, NRRL 832.A2 | Substrain of NRRL 832. Maximum yields of 100 units/ml. | Submerged- production dustry pr summer o |
| *P. chrysogenum*, NRRL 1951.B25 | Substrain of NRRL 1951. Yields in excess of 200 units/ml. Produced higher yields of pencillin in submerged culture than NRRL 832 | Submerged-c production dustry dur latter half 1944 and 1945 |
| *P. chrysogenum*, Stanford 25099 | Substrain of NRRL 1951.B25 resulting from ultraviolet radiation (G. W. Beadle) | Superior submerged-cult production |
| *P. chrysogenum*, Stanford 35217 | Substrain of NRRL 1951.B25 resulting from ultraviolet radiation (G. W. Beadle) | Limited produ by submerg culture in in try |
| *P. chrysogenum*, X-1612 | Mutation of NRRL 1951.B25 induced by X rays by M. Demerec and associates at Carnegie Institute of Washington. Yields in excess of 500 units/ml. | Submerged-cul production perior strain |
| *P. chrysogenum*, NRRL 1984 | Isolated from soil in Minnesota. Minn. R-13 (C. M. Christensen) | The parent of mercially im tant strains for submerge culture produ tion |
| *P. chrysogenum*, Minn. 9-SS251 | Substrain of Minn. R-13 | Submerged-cult production ( yields) |
| *P. chrysogenum*, NRRL 1984.A | Substrain of NRRL 1984 (Minn. R-13). Greater yields than parent. Yields to 260 units/ml. Best producer of penicillin X | Submerged-cult production in industry |

* Based principally on information contained in articles by K. B. Raper and D Fennell, *Jour. Bact.*, **52**(6): 761 (1946); and K. B. Raper and D. F. Alexander, *Jo* *Elisha Mitchell Sci. Soc.*, **61**(1 and 2): 74 (1945).

TABLE 218. INFORMATION CONCERNING SOME MOLDS USED FOR PENICILLIN PRODUCTION *—(*Continued*)

| ation of mold | History | Use |
|---|---|---|
| *sogenum,*<br>L 1984.N22 | Substrain of NRRL 1984.A, resulting from treatment of conidia with ultraviolet radiation. About one-half of penicillin units are penicillin X | Submerged-culture production of penicillin X |
| *sogenum,*<br>(Wisconsin) | Substrain of *P. chrysogenum* X-1612 resulting from treatment of conidia with ultraviolet light (M. P. Backus and J. F. Stauffer, University of Wisconsin). Yields in excess of 761 units/ml. | Submerged-culture production |

e large yields of penicillin. Strain 49-133 was reported by Ander-
d associates (1953) to be a superior strain yielding about 99 per
penicillin G.

LATION MEDIA. In the production of penicillin, it is necessary to
pidly large numbers of spores for inoculation or seeding purposes.
ber of media have been developed for that purpose, of which a few
described.

rding to Moyer and Coghill (1946*a*), good results may be obtained
e following medium:

SPORULATION MEDIUM

| | |
|---|---|
| lycerol | 7.5 g. |
| ane molasses (edible quality as commonly sold at retail) | 7.5 g. |
| orn-steep liquor | 2.5 g. |
| $gSO_4 \cdot 7H_2O$ | 0.050 g. |
| $H_2PO_4$ | 0.060 g. |
| eptone | 5.00 g. |
| aCl | 4.00 g. |
| e-tartrate | 0.005 g. |
| $uSO_4 \cdot 5H_2O$ | 0.004 g. |
| gar | 2.50 g. |
| istilled water to make | 1.0 liter |

going medium may be solidified by adding sufficient agar to bring
l amount to at least 15 to 25 g. per liter.

also described an alternate method for producing mold spores.
method, fresh whole-wheat bread (free of mold inhibitors) is cut
m. cubes and steam-sterilized in shallow layers in Erlenmeyer
The cooled bread is heavily inoculated with mold spores and
d at 25 to 27°C. for 4 to 5 days. After the bread cultures have
dried, they may be stored at 40°C. for as long as 2 weeks satis-
. The spore-covered bread may be crushed usually to a powdery
er 6 to 7 days and used for inoculation purposes. As an alterna-
nay be mixed with 3 to 4 volumes of a mixture of equal parts of
whole-wheat flour and finely ground oat hulls.

Wheat bran moistened with a 2 per cent solution of corn-steep
may also be used for producing spores, according to Moyer and (
(1946*b*).

A sporulation medium of the following composition has been dev
by Koffler and his associates (1946).

| | | | |
|---|---|---|---|
| Agar | 15.00 g. | $KH_2PO_4$ | |
| Sugar-beet molasses | 5.00 g. | $MgSO_4 \cdot 7H_2O$ | |
| Peptone | 5.00 g. | Distilled water to make | |
| NaCl | 4.00 g. | | |

Foster and his coworkers (1945) used the following sporulation me

| | | | |
|---|---|---|---|
| Sucrose or brown sugar | 20 g. | $MgSO_4 \cdot 7H_2O$ | |
| $NaNO_3$ | 6 g. | $CaCl_2$ | 2 |
| $KH_2PO_4$ | 1.5 g. | Water, tap or distilled | 1 |

NOTE: Aeration and agitation are supplied by shaking the flasks containing
inoculated medium on a rotary shaker.

INOCULATION METHODS. There are a number of methods used to ino
late or seed the various media employed in the manufacture of p
cillin, a few of which will be mentioned here. In surface-culture meth
the surface of the medium is inoculated with dry spores, either alone
mixed with such materials as wheat flour or finely ground oat hulls, as
been described by Moyer and Coghill. The spore material is applied
means of the inoculation loop, the spatula, the atomizer, or other meth
the surface being covered as uniformly as possible.

The sporulation medium may be heavily inoculated with spores a
then thoroughly mixed. It should, of course, be exposed in shall
layers.

In submerged-culture methods, the production medium may be ino
lated with dry spores, by ungerminated spores in suspension, or by pel
inocula. Suspensions of ungerminated spores may be prepared in ster
0.1 per cent soap solutions, in sterile water containing 100 p.p.
(1:10,000) of sodium lauryl sulfonate, in sterile aqueous solutions co
taining some other wetting agent, which must be nontoxic to the mo
and penicillin, or by other means. The wetting agents aid in obtaini
more uniform suspensions than can be secured with ordinary sterile wat
The suspensions are introduced into the production medium by means
pipettes, spray guns, bazookas, or other means. The suspensions a
uniformly mixed with the medium by agitation and aeration.

Pellet inocula are those obtained by growing mycelium from mo
spores under submerged conditions. The pellets are commonly used f
inoculation purposes 2 or 3 days after the medium has been seeded wi
spores. Time is saved in the production stage by using pellet inoculation

In a typical procedure for the production of penicillin, the spores
*P. chrysogenum* on agar slants are used to seed a germination medium
flasks (Fig. 123). After a suitable period of incubation on the shaker t

23. Shaker table containing flasks in which molds are being grown for seed ses. (*Courtesy of the Upjohn Company, Kalamazoo, Mich.*)

Transfer rooms. Mold growth from culture flasks is being transferred to metal containers for use in charging seed tanks. (*Courtesy of the Upjohn* *y, Kalamazoo, Mich.*)

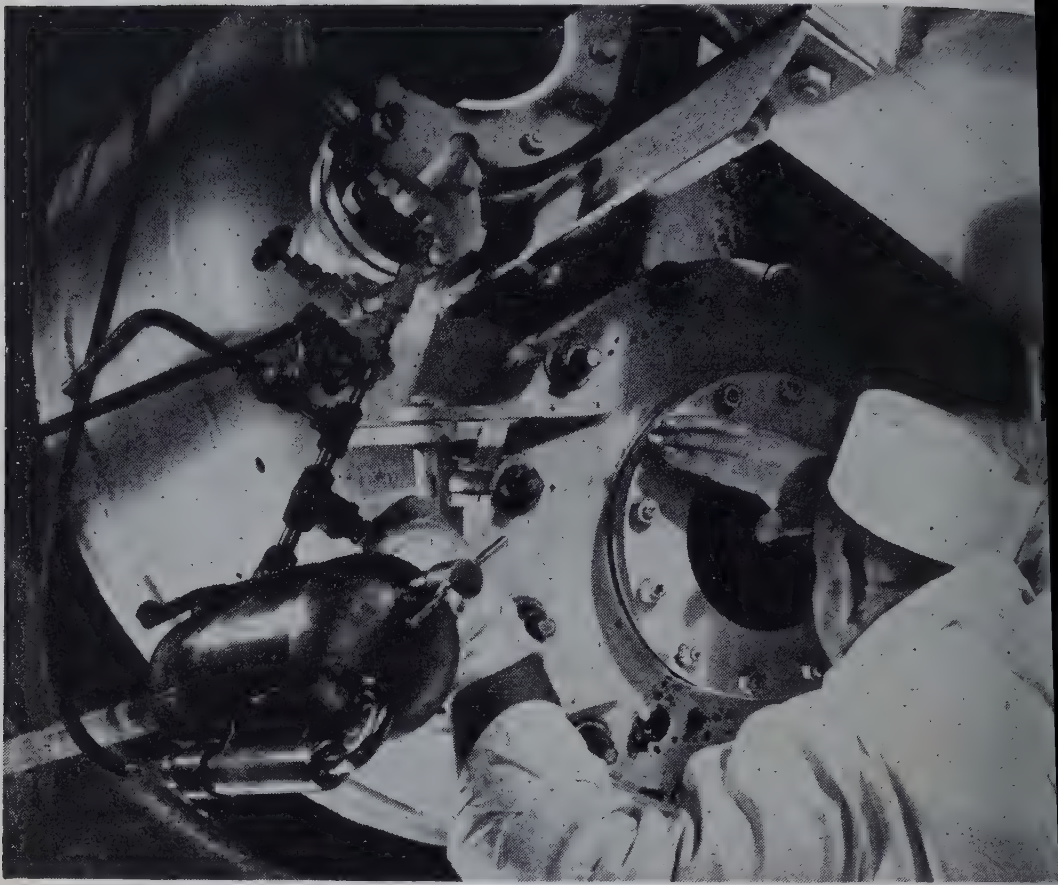


FIG. 125. Charging the seed tank. The penicillin or streptomycin cultures are fo from the metal flasks into 500-gal. seed tanks by means of sterile air under press (*Courtesy of the Upjohn Company, Kalamazoo, Mich.*)

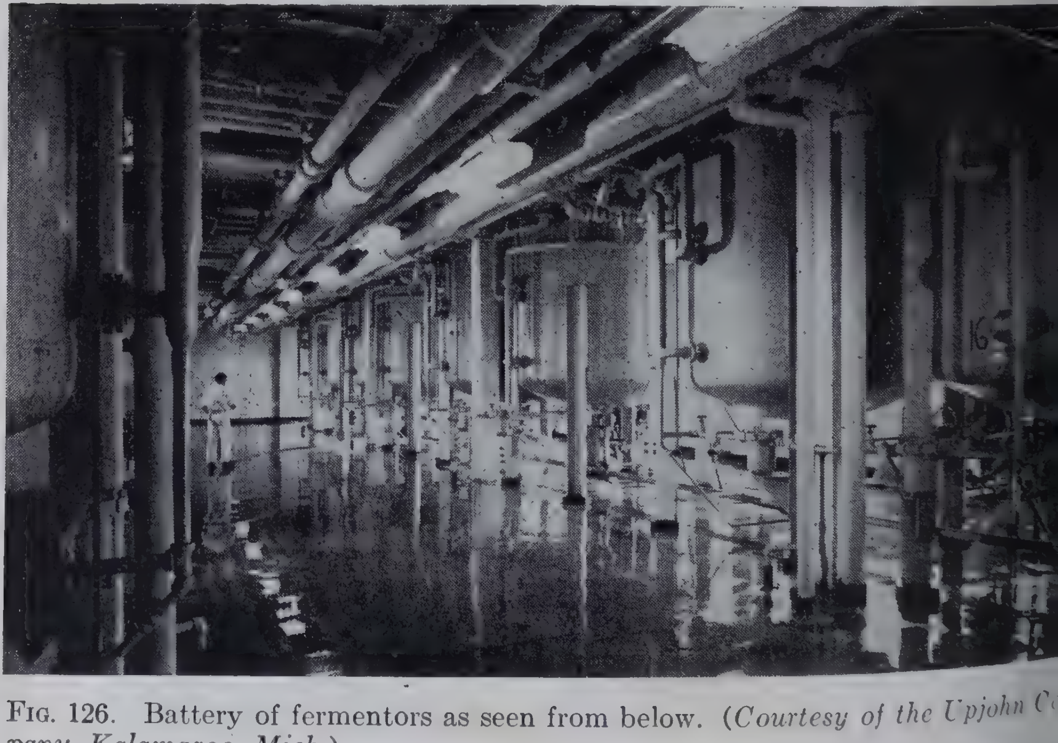


FIG. 126. Battery of fermentors as seen from below. (*Courtesy of the Upjohn C pany, Kalamazoo, Mich.*)

ents are transferred from the shaker flasks to special metal containers . 124). The special container is aseptically attached to the seed (Fig. 125), and the mold suspension is forced into it through the f sterile air under pressure. The mold from the seed tank is used as rter for the fermentors (Fig. 126).

*w Materials.* In the selection of the raw materials for the produc- of penicillin, it is essential to consider the nutritive requirements of old, the conditions for the optimum accumulation of penicillin, and ubsequent extraction and purification of the penicillin.

BON SOURCES. Lactose is a very satisfactory source of carbon for roduction of penicillin when used in a concentration of 6 per cent er and Coghill, 1946*a*). At 3 per cent levels, lactose, cornstarch, and lextrin are of similar value for producing the antibiotic. When slow g rates are used, glucose and sucrose may be employed as satis- y substitutes for lactose (Soltero and Johnson, 1953; Hosler and on, 1953; Davey and Johnson, 1953). Glycerol and sorbitol are r sources of carbon.

ROGEN SOURCES. A number of compounds may be used as sources rogen, including sodium nitrate; ammonium sulfate, ammonium e, and ammonium lactate (Soltero and Johnson, 1953); corn-steep ; and other materials.

ERAL SOURCES. The mineral requirements for mold growth and lin production have been studied by Pratt (1945), Moyer and l (1946*a*, *b*), Stone and Farrell (1946), Foster et al. (1946), Hos- l Johnson (1953), and others.

ain elements (phosphorus, potassium, magnesium, sulfur, zinc, and ) appear to be necessary or beneficial for the production of penicil- some of them are to be found in the corn-steep liquor. Potassium osphorus are customarily supplied as potassium dihydrogen phos- magnesium and sulfur, as magnesium sulfate ($MgSO_4 \cdot 7H_2O$). d copper, when added to the medium, are usually supplied as the . According to Moyer and Coghill (1946*b*), the addition of zinc ganese ions to a medium containing corn-steep liquor is optional. sed, they are supplied as the sulfates.

-steep liquor appears to contain adequate quantities of $KH_2PO_4$ $gSO_4 \cdot 7H_2O$, according to Moyer and Coghill (1946*a*) and Foster ociates. However, small amounts of these salts may be added to duction medium.

(1945) studied the influence of various proportions of $KH_2PO_4$, $\cdot 7H_2O$, and $NaNO_3$ on penicillin production by *P. notatum* in ontaining lactose, corn-steep liquor, zinc sulfate, and phenylacetic e found that the absolute concentrations of these salts in the best were $KH_2PO_4$, 0.019 *M*; $MgSO_4 \cdot 7H_2O$, 0.002 *M*; and $NaNO_3$, (a total molar concentration of 0.040).

STEEP LIQUOR. The use of corn-steep liquor in the fermentation greatly increases the yields of penicillin. This fact was demon- in respect to the surface-culture and submerged-culture produc- enicillin by Moyer and Coghill (1946*a*, *b*).

Corn-steep liquor, or steep water, is a nutrient extract obtained the manufacture of starch and other corn products. The role material in the production of penicillin has been studied by Bowde Peterson (1946), who reported that corn-steep-liquor samples from ent producers showed the following analyses:

| | Per cent | | P |
|---|---|---|---|
| Solids ........................ | 40–60 | Amino nitrogen * ............. | |
| Lactic acid * .................. | 12–27 | Reducing sugars, as glucose *... | |
| Total nitrogen * ............... | 7.4–7.8 | Ash * ........................ | |

* On a dry basis.

There is considerable variation in penicillin yields from samples different manufacturers and in lots from the same manufacturer, ac ing to Bowden and Peterson.

The optimum concentration of corn-steep solids in shake cultures found by Bowden and Peterson to be 2 per cent for both *P. not* NRRL 832 and *P. chrysogenum* X-1612. However, a concentration per cent was observed to be optimum when larger volumes of me were used with aeration and agitation.

Cuban high-test molasses, steffanized molasses A and B, Bacto tone, Difco yeast extract, solubilized liver, asparagus-butt juice, g juice powder, rice steep, cottonseed-meal extract, ground liver, and infusion were found to be of little value as corn-steep-liquor substit Although meat-scraps meal was somewhat superior to most of the going substances, it was inferior to corn-steep liquor, according to Bow and Peterson.

Foster and associates (1946) reported that the stimulatory effect corn-steep liquor were due in part to arginine, histidine, and glutamic and possibly to phenylacetic acid derivatives.

COTTONSEED MEAL. Cottonseed meal was found by Foster and associates (1946) to be equal to or superior to corn-steep liquor for p cillin production by *P. chrysogenum* Demeric X-1612 and *P. chrysoge* Wisconsin Q176. It stimulated production by *P. notatum* NRRL 832 was inferior to corn-steep liquor for this strain.

PRECURSORS. Precursors are substances used in the production antibiotics to increase the yield or to encourage the formation of a ticular type of compound. Through the incorporation of suitable acyl chains, a large number of related penicillins may be produced.[1] The ef of phenylacetic acid derivatives on the formation of penicillin by m has been studied by Moyer and Coghill (1947), Higuchi et al. (19 Stone and Farrell (1946), and many others. Arnstein and Grant (19 Pratt and Dufrenoy (1953), and others have reviewed this import subject.

Higuchi and his associates (1946) determined the effect of ad phenylacetic acid derivatives on penicillin production by *P. chrysogen* Q176. Information concerning their results and the fermentation m

---

[1] *Research Today,* **5**: 8 (1949).

E 219. EFFECT OF PHENYLACETIC ACID DERIVATIVES ON PENICILLIN PRODUCTION [a]

| Compound added [c] | Age of fermentation, hr. | Total penicillin, units/ml. | Percentage composition of penicillin, in yields | | |
|---|---|---|---|---|---|
| | | | G | X [d] | K |
| None | 42 | 94 | 13 | .. | 87 |
| | 66 | 233 | 14 | .. | 87 |
| | 75 | 264 | 12 | .. | 88 |
| β-Phenylethylamine | 42 | 117 | 91 | .. | 9 |
| | 66 | 272 | 76 | .. | 24 |
| | 90 | 435 | 66 | .. | 34 |
| Phenylacetic acid [e] | 42 | 169 | 88 | .. | 12 |
| | 66 | 333 | 79 | .. | 21 |
| | 75 | 387 | 74 | .. | 26 |
| Phenylacetamide | 42 | 119 | 91 | .. | 9 |
| | 66 | 194 | 69 | .. | 31 |
| | 90 | 384 | 57 | .. | 43 |
| None | 60 | 161 | 44 | 3 | 53 |
| | 108 | 569 | 29 | 1 | 70 |
| β-Phenylethylamine | 60 | 267 | 93 | 2 | 5 |
| | 108 | 726 | 78 | 5 | 17 |
| Phenylacetamide [e] | 60 | 335 | 103 | 1 | −14 |
| | 108 | 616 | 78 | 2 | 20 |
| Phenylacetic acid [e] | 60 | 448 | 109 | 3 | −12 |
| | 108 | 673 | 76 | −1 | 25 |
| p-Hydroxyphenylacetic acid | 60 | 195 | 57 | 18 | 25 |
| | 108 | 422 | 42 | 11 | 47 |
| p-Hydroxyphenylacetic acid [f] | 60 | 209 | 39 | 26 | 35 |
| | 108 | 462 | 34 | 10 | 56 |
| None | 49 | 638 | 30 | .. | 70 |
| Phenylacetic acid [f] | 24 | 216 | 35 | .. | 65 |
| | 60 | 1000 | 67 | .. | 33 |
| | 72 | 1045 | 77 | .. | 23 |

Iiguchi, F. G. Jarvis, W. H. Peterson, and M. J. Johnson, *Jour. Am. Chem.*
(8): 1669–1670 (1946). Copyright 1946 by the American Chemical Society
inted by permission of the copyright owner.

1 and 2 were carried out in 500-ml. Erlenmeyer flasks containing 85 ml. (run
ml. (run 2) of medium. The medium used in run 1 consisted of the follow-
tituents in grams per liter: lactose, 22.5; glucose, 7.5; ammonium lactate,
assium dihydrogen phosphate, 2.0; magnesium sulfate, 0.25; ferrous sulfate,
pper sulfate, 0.005; zinc sulfate, 0.02; aluminum chloride, 0.00027; potassium
ate, 0.000053. The medium used in run 2 contained the following constituents
per liter: corn-steep-liquor solids, 30; lactose, 30; calcium carbonate, 10.
and 4 were carried out in aerated and agitated tanks. The volume of medium
220 liters. The medium used in run 3 contained in grams per liter: steep-
lids, 40; lactose, 40; calcium carbonate, 10; sodium sulfate, 1.0. The medium
un 4 was the same as that in run 3 except that the steep-liquor concentration
. per liter.

compounds were added at a level of 0.5 g. per liter, and were added before
ion unless otherwise stated.

the differential assay results were calculated as a mixture of G and K only,
is given for X.

ompound was added 24 hr. after inoculation.

henylacetic acid was added in six equal portions, at 0, 12, 24, 36, 48, and 60 hr.

used by them is summarized in Table 219. Study of this table the following conclusions: *P. chrysogenum* Q176 produced large tions of penicillin K in the absence of phenylacetic acid derivati high as 88 per cent in one run). The addition of phenylacetic acid tives greatly increased the proportions of penicillin G produced, larly during the early hours of the fermentation. The over-all were also increased by the phenylacetic acid derivatives, especi phenylacetic acid. Corn-steep liquor, a source of these derivative wise increased the proportion of penicillin G and the total amo penicillin formed. Para-hydroxyphenylacetic acid increased the ties of penicillin X produced, but, according to Higuchi and his a ates, it appeared to be less readily available to the mold than p acetic acid.

Since phenylacetic acid is toxic to the molds under certain cond for example, at low pH values, it is necessary to observe certain p tions in connection with its use. Moyer and Coghill (1947) found the toxicity could be overcome by increasing the pH of the medium t 5.8 before inoculation or by adding the phenylacetic acid to the c after the pH had risen above the critical value.

Satisfactory results have been obtained in flask fermentations by separate additions of 0.05 per cent of phenylacetic acid at 12-hr. int (Anderson et al., 1953).

Straight-chain fatty acids increase the yield of penicillins, when a to the production medium; however, they tend to favor the formati less desirable penicillins.

Amino acids, such as L-cystine, L-cysteine, and valine, appear important in the synthesis of the beta-lactamthiazolidine ring syste the penicillins (Arnstein and Grant, 1954*a*, *b*, 1956).

*Conditions of Fermentation.* Under this heading may be consi such factors as temperature, pH, aeration, agitation, the use of anti agents, and the prevention of the contamination of the medium.

TEMPERATURE. A temperature of about 24°C. appears to be opti for this fermentation.

*pH.* Control of the pH during the fermentation is essential, fo high or too low a pH may result in considerable losses in penic According to Moyer and Coghill, the loss of penicillin may be substa outside the range of pH 5 to 7.5. In a normal fermentation, the pH from 4 to around 8. Such factors as the fermentation of the corn-s liquor with the utilization of the lactic acid, the utilization of the ca from amino acids with the liberation of ammonia, and the autolys the mold account for the rise in pH to 8 or above.

The pH of the medium is influenced by the kind and amount of ca hydrate present, the amount of corn-steep liquor, the presence of buf and other factors.

Carbohydrates are generally fermented with the production of organic acids. Some carbohydrates, such as glucose, are fermented idly and tend to lower the pH level; others, such as lactose, are mented more slowly, and consequently the pH changes more slo concentrations of fermentable sugars tend to retard the pH rise in nedium. In a submerged medium containing corn-steep liquor, se, and calcium carbonate, the mold utilizes the carbon from the ger.-containing portions of the corn-steep liquor in preference to the se at the beginning of the fermentation, according to Johnson.

gh yields of penicillin may be obtained from glucose and sucrose these sugars are fed into the production medium at a slow rate, continuously or intermittently. Sugar feed rates of 0.03 to 0.07 per per hour were found to be optimum by Hosler and Johnson (1953).

fer agents tend to maintain the pH within a range favorable for llin production. Calcium carbonate is widely used for this purpose. added to the medium, it raises the initial pH and tends to resist the g of the pH to high final values. Calcium carbonate is not used in e-culture production, for it decreases the growth of the mold and eld of penicillin.

ATION. Adequate aeration is essential for optimum yields of penifor the molds are aerobic organisms and energy for synthetic procincluding the building of cells, is aided by the presence of available n. In shake-flask cultures, aeration is supplied by the rotary n of the shaker; in the deep tank, it is supplied by forcing air h the medium, by means of agitation, and occasionally by the use under pressure.

requirements for a given tank will depend upon the diameter of nk, the depth of the medium, the nature of the medium, the design aerating device (sparger), the design of the agitator, and other They must be determined for each installation.

niak and coworkers found an aeration rate of 1 volume of air per for each volume of medium in 100-gal. tanks holding 200 liters um to be optimum. Taylor (1947) described a commercial process n not less than 400 cu. ft. of air per minute was blown into the end mentation tank of approximately 5,000-gal. capacity through a

URE. The use of pressure in tanks to increase the effectiveness ion is common practice. Stefaniak and his associates usually out fermentations at a tank pressure of 20 lb. per sq. in. They hat reduction of the pressure to 2 lb. per sq. in. did not affect the ation adversely but that a tank pressure of 40 lb. per sq. in. in reduced yields of penicillin.

TION. The use of the proper type of stirrer and the correct speed prove the yield of penicillin at a constant aeration rate (Stefaniak 946).

OAM AGENTS. A number of chemical agents have been suggested o prevent excessive foam formation during the production of penisubmerged-culture methods. Prevention of excessive foaming n better yields of penicillin.

yl citrate was used as an antifoam agent by Foster and his rs.

iak and his associates found 3 per cent octadecanol dissolved in

lard oil to be the most useful of the antifoam agents tested b
which included lard, lard oil, 3 per cent octadecanol in lard oil,
oil, 3 per cent octadecanol in soybean oil, Vegifat Y, and Nopco d
Lard, lard oil, and 3 per cent octadecanol in lard oil were found to
toxic in concentrations of 0.1 to 1.0 per cent. Although Nopco d
and Vegifat Y were the most effective foam breakers, they were l
the most toxic of the antifoam agents examined. Three per cent o
anol in soybean oil was toxic in shake-flask but not in tank ferment

Alkaterge C in lard oil has been used satisfactorily (Davey and
son, 1953).

Antifoam agents, in general, reduced the pH levels of the ferme
media progressively as their concentrations were increased.

PREVENTION OF CONTAMINATION. In no fermentation is it more es
to prevent contamination of the medium than in the penicillin fern
tion. Contamination usually produces rapid destruction of penicill
order to prevent contamination, it is necessary to sterilize all fermen
tanks, pipe lines, and equipment with which the penicillin may com
contact. Steam is the agent used for sterilization of facilities and
Antifoam agents must be sterilized before aseptic addition to the me
Starters must be added aseptically. All air used for aeration mu
sterilized.

*Production of Penicillin on Synthetic Media.* Although media co
ing corn-steep liquor and lactose have been widely used for prod
penicillin, Hosler and Johnson (1953) showed that synthetic media
taining glucose and no corn-steep liquor may be used for obtainin
antibiotic in high yields. They obtained as high as 1,800 units pe
of penicillin from *P. chrysogenum* Q176 in 30-liter fermentors (ref
Fig. 127).

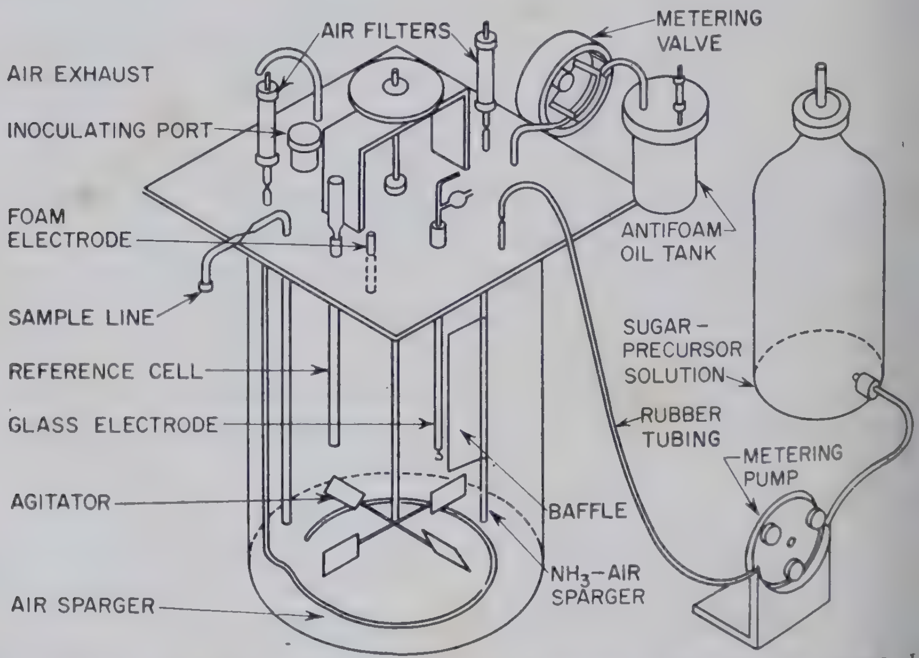
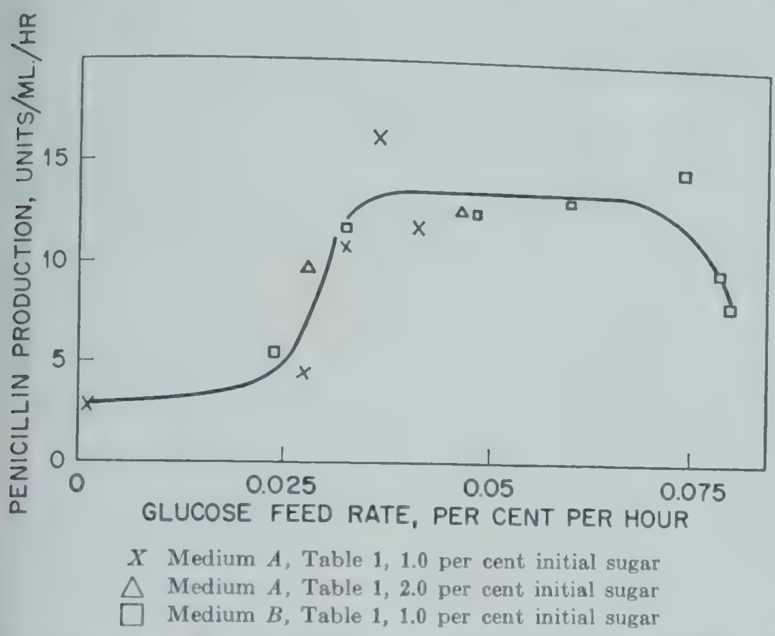

AIR FILTERS
METERING VALVE
AIR EXHAUST
INOCULATING PORT
FOAM ELECTRODE
ANTIFOAM OIL TANK
SAMPLE LINE
SUGAR-PRECURSOR SOLUTION
REFERENCE CELL
RUBBER TUBING
GLASS ELECTRODE
METERING PUMP
AGITATOR
BAFFLE
NH3-AIR SPARGER
AIR SPARGER

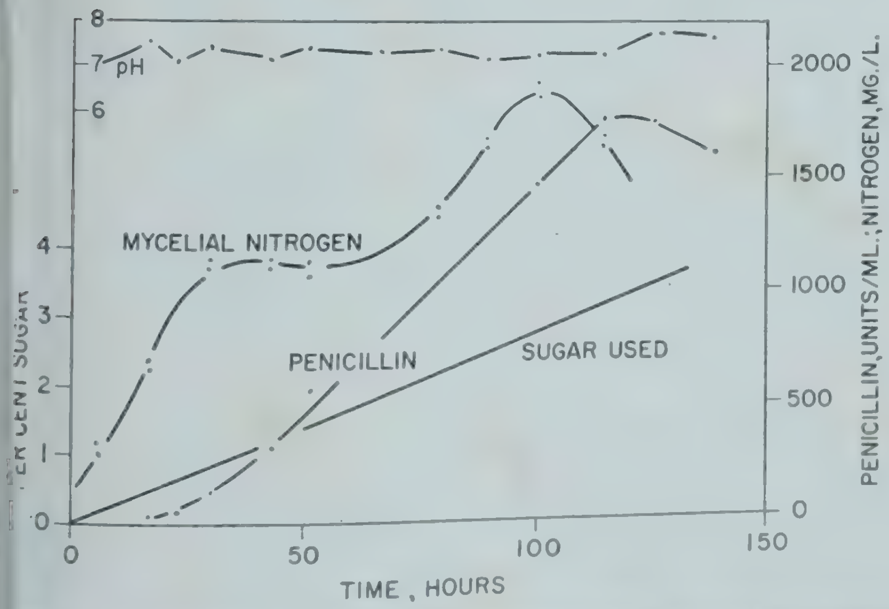
FIG. 127. Thirty-liter-fermentor assembly. [*Courtesy of P. Hosler and M. J. J*
*son, Ind. Eng. Chem.,* 45: 871 (1953). *Copyright* 1953 *by the American Che*
*Society and reprinted by permission of the copyright owner.*]

imum conditions for production included the use of 1 per cent of glucose, the maintenance of a pH plateau of 7.4, and the use of feed rates of 0.03 to 0.07 per cent per hour (Fig. 128).

synthetic medium (medium B), which gave satisfactory results, ned, in grams per liter, the following ingredients: glucose, 10.0;



Optimum feed rate for penicillin production. pH plateau, 7.4. [*Courtesy*
*sler and M. J. Johnson, Ind. Eng. Chem.*, **45**: 871 (1953). *Copyright* 1953
*American Chemical Society and reprinted by permission of the copyright*



Chemical changes in a continuous-feed glucose fermentation without sepa-
a phase (medium B). Glucose feed rate, 0.0294 per cent per hour. [*Cour-*
*Hosler and M. J. Johnson, Ind. Eng. Chem.*, **45**: 871 (1953). *Copyright*
*e American Chemical Society and reprinted by permission of the copy-*
*r.*]

$(NH_4)_2SO_4$, 0.4; $KH_2PO_4$, 7.5; $Na_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2$ $FeSO_4 \cdot 7H_2O$, 0.18; $MnSO_4 \cdot 4H_2O$, 0.05; $ZnSO_4 \cdot 7H_2O$, 0.05; 0.008; $CaCl_2$, 0.05; and $CaSO_4$, 2.0. The glucose was separately st in distilled water and added at the time of inoculation. Addition cose was fed to the medium during the production phase at the rate fied above.

The chemical changes which took place in medium B with cont glucose feed are shown in Fig. 129.

*Commercial Production.* High-yielding strains of *P. chryso* such as those described by Anderson et al. (1953), are used to p penicillin under submerged-culture conditions. A flow chart for th duction of penicillin is shown in Fig. 130; an interior view of a fer is shown in Fig. 131.

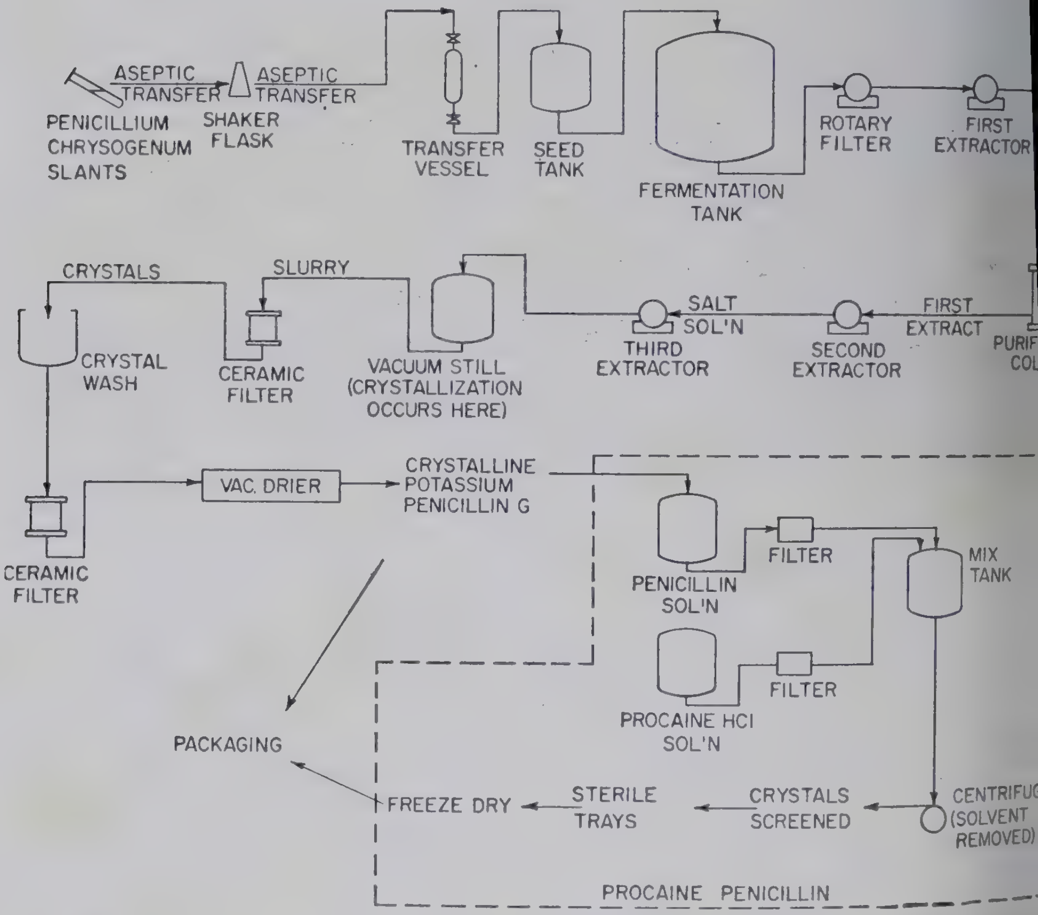

ASEPTIC TRANSFER
ASEPTIC TRANSFER
PENICILLIUM CHRYSOGENUM SLANTS
SHAKER FLASK
TRANSFER VESSEL
SEED TANK
FERMENTATION TANK
ROTARY FILTER
FIRST EXTRACTOR
CRYSTALS
SLURRY
CRYSTAL WASH
CERAMIC FILTER
VACUUM STILL (CRYSTALLIZATION OCCURS HERE)
SALT SOL'N
THIRD EXTRACTOR
SECOND EXTRACTOR
FIRST EXTRACT
PURIF
COL
VAC. DRIER
CRYSTALLINE POTASSIUM PENICILLIN G
CERAMIC FILTER
PENICILLIN SOL'N
FILTER
MIX TANK
FILTER
PROCAINE HCl SOL'N
PACKAGING
FREEZE DRY
STERILE TRAYS
CRYSTALS SCREENED
CENTRIFU (SOLVENT REMOVED)
PROCAINE PENICILLIN

Fig. 130. Penicillin flow chart. (*Courtesy of the Upjohn Company, Kalam Mich.*)

The commercial manufacture of penicillin has been described by Fl et al. (1949), by Pratt and Dufrenoy (1953), by Campbell (1953), an others.

*Laboratory Methods.* Laboratory methods for the production of p cillin by surface-culture and submerged-culture techniques have reviewed by Prescott and Dunn (1949) and others.

Interior view of fermentor. [*Courtesy of T. H. M. Taylor; Chem. Eng.* (4): 155 (1947).]

## E ISOLATION AND PURIFICATION OF ANTIBIOTICS

does not permit an extended discussion of this important aspect ubject. However, the reader is referred for additional detail to s of Florey et al. (1949), Pratt and Dufrenoy (1953), and others.

bjective of isolation is to separate the comparatively minute of antibiotic from the producing cells and the large volume of edium.

rst step in the recovery process is the removal of mycelium or filtration (Fig. 132) or centrifuging. Continuous-vacuum filters, ing filters, basket centrifuges, or other equipment may be used, g on the nature of the cells.

cond important step is to remove the antibiotic from the spent n medium by solvent extraction, adsorption or precipitation, or a ion of methods.

t extraction may be accomplished in countercurrent extractors ) such as the Podbielniak, in towers, in flasks, in the Craig dis-

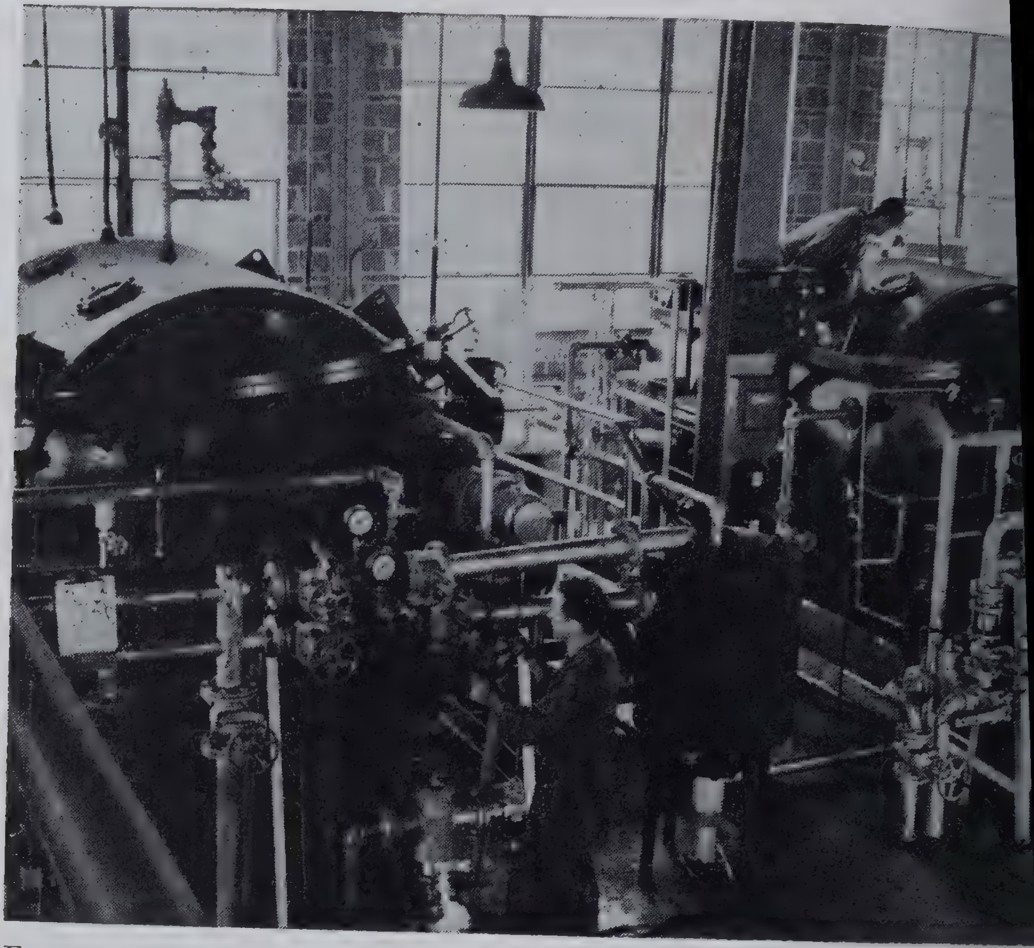


Fig. 132. Filtration units used to remove mycelium from the fermented solu
*(Photo from Merck & Co., Inc.)*

tribution system, or otherwise, depending on the volume of material the objective. Amyl acetate, ether, chloroform, acetone, and other vents may be used for the extraction, according to the solubilities of antibiotic, practicability, etc. Temperature and pH control are extrem important factors in securing high yields of the antibiotics.

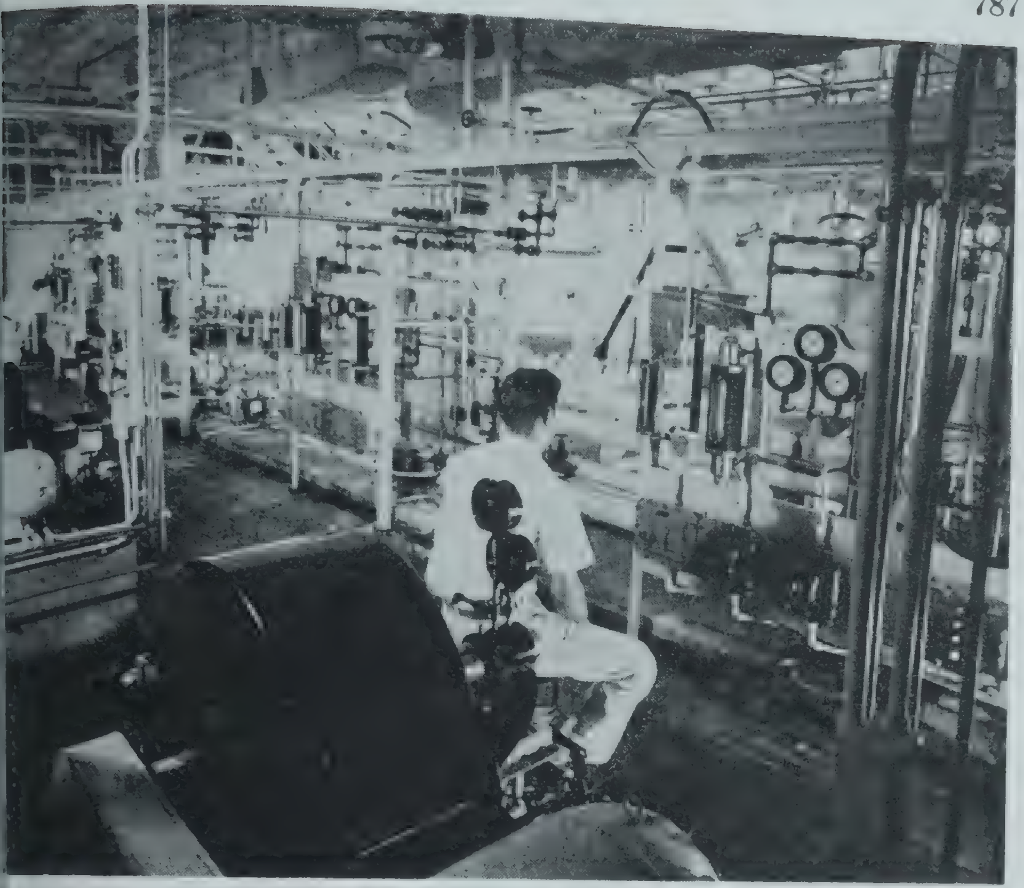
Adsorption may be carried out on active carbon, ion-exchange re or other materials.

Purification is accomplished by additional solvent extraction, by tillation, by sublimation, by column chromatography, or by other me depending on the nature of the antibiotic, cost, and other factors. product may be sterilized and rendered free of pyrogens (fever-produc substances) by filtration through Seitz, molecular, or other bacte filters of a suitable nature.

A basic flow sheet for the recovery of antibiotics is shown in Fig. 13

## TESTING, ASSAYING, AND CERTIFICATION

The official methods for testing, assaying, and certifying antibiotics a antibiotic-containing drugs are described by the Division of Antibioti Bureau of Biological and Physical Sciences, Food and Drug Administ tion, Department of Health, Education and Welfare, in Vol. I, Parts I



. View of extraction section in antibiotics plant. Extractors in foreground,
tanks in the background. (*Courtesy of the Upjohn Company, Kalamazoo,*

. Part 141 is concerned with testing and assaying; Part 146, with
tion.

s are carried out to determine the potency of the antibiotic and
sterility, toxicity, pyrogenicity, moisture, and others items, as
required.

and Randall (1955) have discussed the testing and assaying of
cs, and their book is highly recommended.

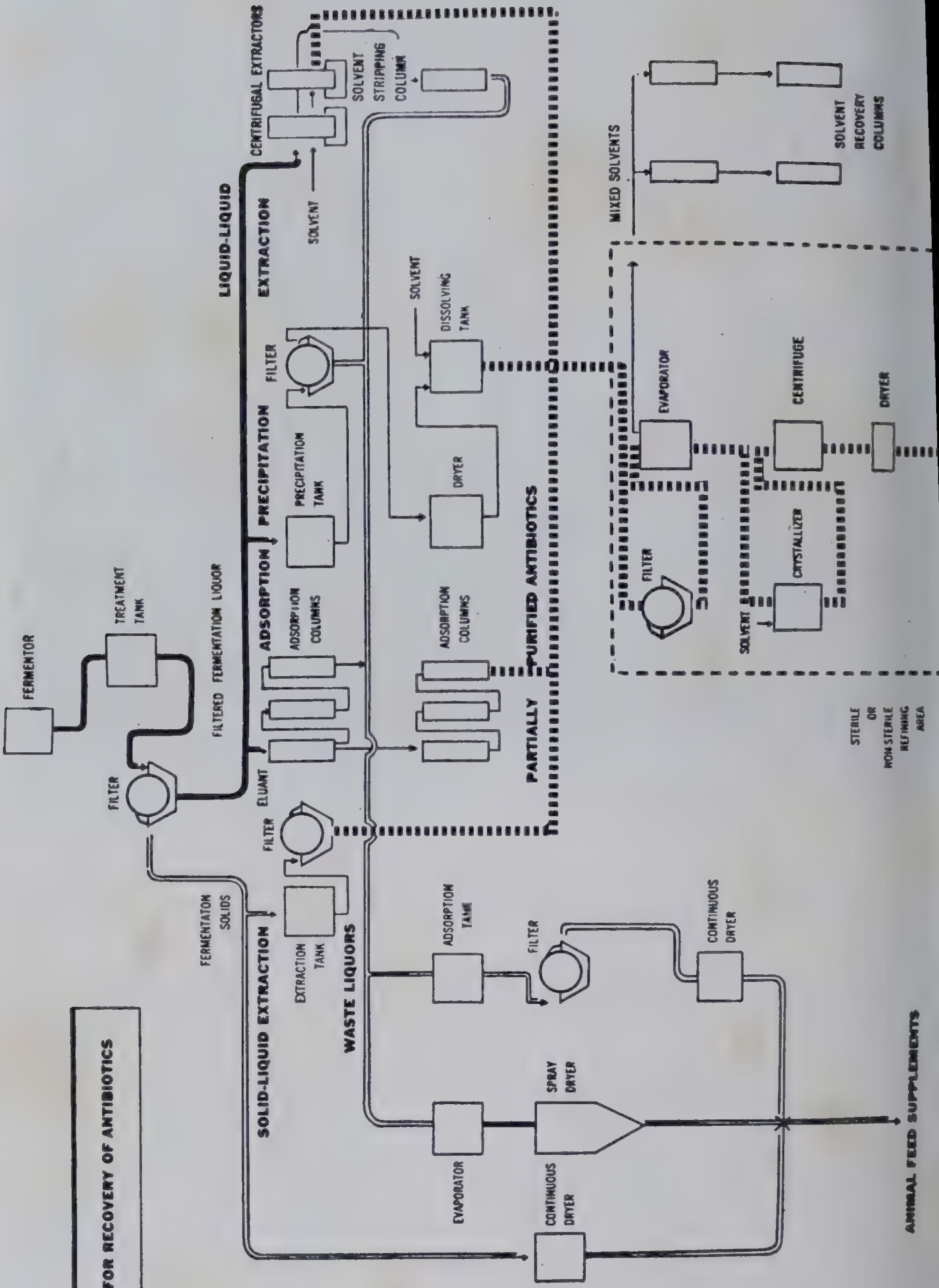
ach and Levine (1953) have described test procedures for many
mmercially produced antibiotics.

nder-plate method for determining the potency of penicillin is
d below. However, the reader is urged to consult one of the
es cited above for additional detail.

**der-plate Method.** This method has been widely used for deter-
he potency of penicillin and, as described below, is the standard
by the Food and Drug Administration for sodium, calcium, and
n penicillins.

st, in brief, consists of adding 21 ml. of a nutrient agar to a
tri dish of greater depth than that ordinarily used for bacterio-
udies, allowing the agar to harden, adding 4 ml. of a second type
t agar which has been seeded with the test organism, spreading
d agar uniformly over the surface of the base agar, permitting it

BASIC FLOWSHEET FOR RECOVERY OF ANTIBIOTICS


FERMENTOR
TREATMENT TANK
FILTER
FILTERED FERMENTATION LIQUOR
FERMENTATON SOLIDS
SOLID-LIQUID EXTRACTION
EXTRACTION TANK
FILTER
ELUANT
ADSORPTION
ADSORPTION COLUMNS
PRECIPITATION
PRECIPITATION TANK
FILTER
LIQUID-LIQUID EXTRACTION
SOLVENT
CENTRIFUGAL EXTRACTORS
SOLVENT STRIPPING COLUMN
SOLVENT
DISSOLVING TANK
DRYER
ADSORPTION COLUMNS
PARTIALLY PURIFIED ANTIBIOTICS
WASTE LIQUORS
ADSORPTION TANK
FILTER
CONTINUOUS DRYER
EVAPORATOR
SPRAY DRYER
CONTINUOUS DRYER
ANIMAL FEED SUPPLEMENTS
MIXED SOLVENTS
SOLVENT RECOVERY COLUMNS
EVAPORATOR
CENTRIFUGE
DRYER
FILTER
CRYSTALLIZER
SOLVENT
STERILE OR NON-STERILE REFINING AREA

idify, dropping four sterile cylinders onto the agar, filling the
lers with suitable dilutions of the working standard of penicillin and
sample of penicillin being assayed, incubating the plates at 37°C.
to 18 hr., measuring the diameters of the zones of inhibition,
g certain calculations with the data obtained, and determining the
y by reference to special charts.

materials required for the test are the test organism, three kinds
dia, petri dishes, porcelain covers for the petri dishes, cylinders
, and suitable dilutions of the working standard of penicillin and of
nple of penicillin being tested.

*Organism.* The test organism is *Staphylococcus aureus* P.C.I.
or A.T.C.C. 6538P.

*ure Media.* The ingredients used in the preparation of the culture
must meet the requirements of the U.S.P. or N.F. However, the
may be prepared from dehydrated mixtures and distilled water,
d that they have the same composition when reconstituted. Like-
minor modifications of the individual ingredients are permissible
esulting media possess growth promoting properties at least equal
nedia described."

trient agar medium of the following composition is used for carry-
test organism and for the seed layer, which may be designated as
1:

| | |
|---|---|
| Peptone | 6.0 g. |
| Pancreatic digest of casein | 4.0 g. |
| Yeast extract | 3.0 g. |
| Beef extract | 1.5 g. |
| Glucose | 1.0 g. |
| Agar | 15.0 g. |
| Distilled water to make | 1,000.0 ml. |
| pH 6.5–6.6 after sterilization | |

ient agar medium of simpler composition, which may be desig-
medium 2, is used for the base layer:

| | |
|---|---|
| Peptone | 6.0 g. |
| Yeast extract | 3.0 g. |
| Beef extract | 1.5 g. |
| Agar | 15.0 g. |
| Distilled water to make | 1,000.0 ml. |
| pH 6.5–6.6 after sterilization | |

rient broth medium, which is used for the preparation of an
of the test organism and which may be designated as medium 3,
ollowing composition:

| | |
|---|---|
| Peptone | 5.0 g. |
| Yeast extract | 1.5 g. |
| Beef extract | 1.5 g. |
| Sodium chloride | 3.5 g. |
| Glucose | 1.0 g. |
| Dipotassium phosphate | 3.68 g. |
| Potassium dihydrogen phosphate | 1.32 g. |
| Distilled water to make | 1,000.0 ml. |
| pH 7.0 after sterilization | |

*Petri Dishes.* These should measure 20 by 100 mm. Porcelai which are glazed on the exterior, are used for the test.

*Cylinders (cups).* The cylinders are made from stainless st have the following measurements: outside diameter, 8 mm. (±0. inside diameter, 6 mm. (±0.1 mm.); and length, 10 mm. mm.).

*The Working Standard.* The working standard of penicillin is from the Food and Drug Administration. It is kept in tightly st vials, which are stored in larger stoppered tubes containing a desiccant and refrigerated at 15°C. (59°F.) or at a lower tem constantly. From 4 to 5 mg. of the working standard is weighe an atmosphere containing 50 per cent or less of relative humid used to make up a stock solution of the desired concentration. 1 per cent phosphate buffer at a pH of 6 is used for diluting the pe The stock solution is stored at a temperature of about 10°C. for only. Working dilutions are prepared from the stock solution.

*Preparation of Plates.* This consists of adding 21-ml. portions o oculated nutrient agar (medium 2) to sterile petri dishes, allowin solidify, adding 4 ml. of seeded agar to the surface of the base agar plate, and dropping the cylinders onto the seeded agar.

The seeded agar is prepared as follows: The test organism, w carried on slants containing medium 1 and transferred about once a is used to inoculate nutrient broth (medium 3). The broth cult incubated at 37°C. for 16 to 24 hr. Two ml. of this culture is thoroughly with a 100-ml. portion of the agar (medium 1), whi been melted and cooled to 48°C. Four ml. of the seeded agar is on the surface of the base agar in each petri dish and spread uni by tilting the dish back and forth.

A suspension of the test organism, which may be used over a pe a week, may be prepared instead of the broth culture for seeding the The test organism is grown on an agar slant (medium 1) at 37° 24 hr. The growth is washed from the surface with 2 ml. of sterile p logical saline solution and transferred to a large surface of aga example, that provided by 300 ml. of agar in a Roux bottle. It i formly distributed over the entire surface of the agar by means of glass beads. The seeded agar is incubated at 37°C. for 24 hr. and at temperature for 24 hr. The growth on the surface of the agar is susp in 50 ml. of sterile physiological saline. The suspension thus obtai standardized "by determining the dilution which will permit 20 pe light transmission through a filter at 6500 angstrom units in a electric colorimeter." Each 100-ml. portion of medium 1 is inoc with 1.5 to 2 ml. of the standardized suspension. Four ml. of the agar is added to each petri dish containing 21 ml. of base ag described above.

The stainless-steel cylinders are sterilized in separate containers, and aseptically added to the surface of the seeded agar by dropping from a height of 0.5 in. with a mechanical guide. Four cylinders are ch plate, separated by about 90° intervals on a radius of 2.8 cm. etri dishes have porcelain covers, glazed on the top.

*paration of the Sample.* A stock solution of the sample that is assayed may be prepared by dissolving it in sterile, distilled

*ay.* A total of four petri dishes, each containing four cylinders, sed for each sample. Two cylinders in each plate are reserved for ons of the working standard and two for dilutions of the sample. ylinder in each plate is filled with a dilution of the working standard ontains 1 unit per ml. of penicillin. A second cylinder in each plate d with a dilution of the working standard that contains 0.25 unit l. A third cylinder in each plate is filled with a dilution of the sam- at contains an estimated 1 unit per ml. A fourth cylinder in each is filled with a dilution of the sample that contains an estimated nit per ml. The plates are placed very carefully in the incubator and stored for 16 to 18 hr. at 37°C. After the incubation period, ameters of the zones of inhibition are measured to the nearest m., by means of a suitable measuring device, such as a colony r containing a measuring scale (in millimeters) etched into the hat supports the dish over the light source.

*mation of Potency and Error.* In order to determine the potency sample, certain values must be calculated from the assay data sed in connection with the chart shown in Fig. 134. The values d are designated as $V$ and $W$. These values may be determined ding together all the $V$ values and the $W$ values, respectively, the plates. The $V$ value for each plate may be determined by the ng formula: $V = (U_L + U_H) - (S_L + S_H)$. The $W$ value for each ay be determined by the following formula:

$$W = (U_H + S_H) - (U_L + S)$$

$S_L$ represent the diameters (in millimeters) of the zones of inhibi- the 1.0-unit and the 0.25-unit dilutions of the standard, respec- and $U_H$ and $U_L$ represent the diameters of the zones of inhibition of responding dilutions of the test sample. After the $W$ and $V$ values en calculated, the point on the chart (Fig. 134) that corresponds e values is located and the potency ascertained from the radial

chart should not be used for determining the potency of a wherein the potency is lower than 50 per cent or higher than 150 nt of the standard. Where the potency varies to this extent, ay should be repeated, using a higher or lower dilution of the

letails concerning the procedure to be followed in estimating the assay, the reader is referred to Part 141, Chap. I—Food and dministration, Title 21—Food and Drugs.

METHODS. Table 220 presents some data on assay methods for ics.

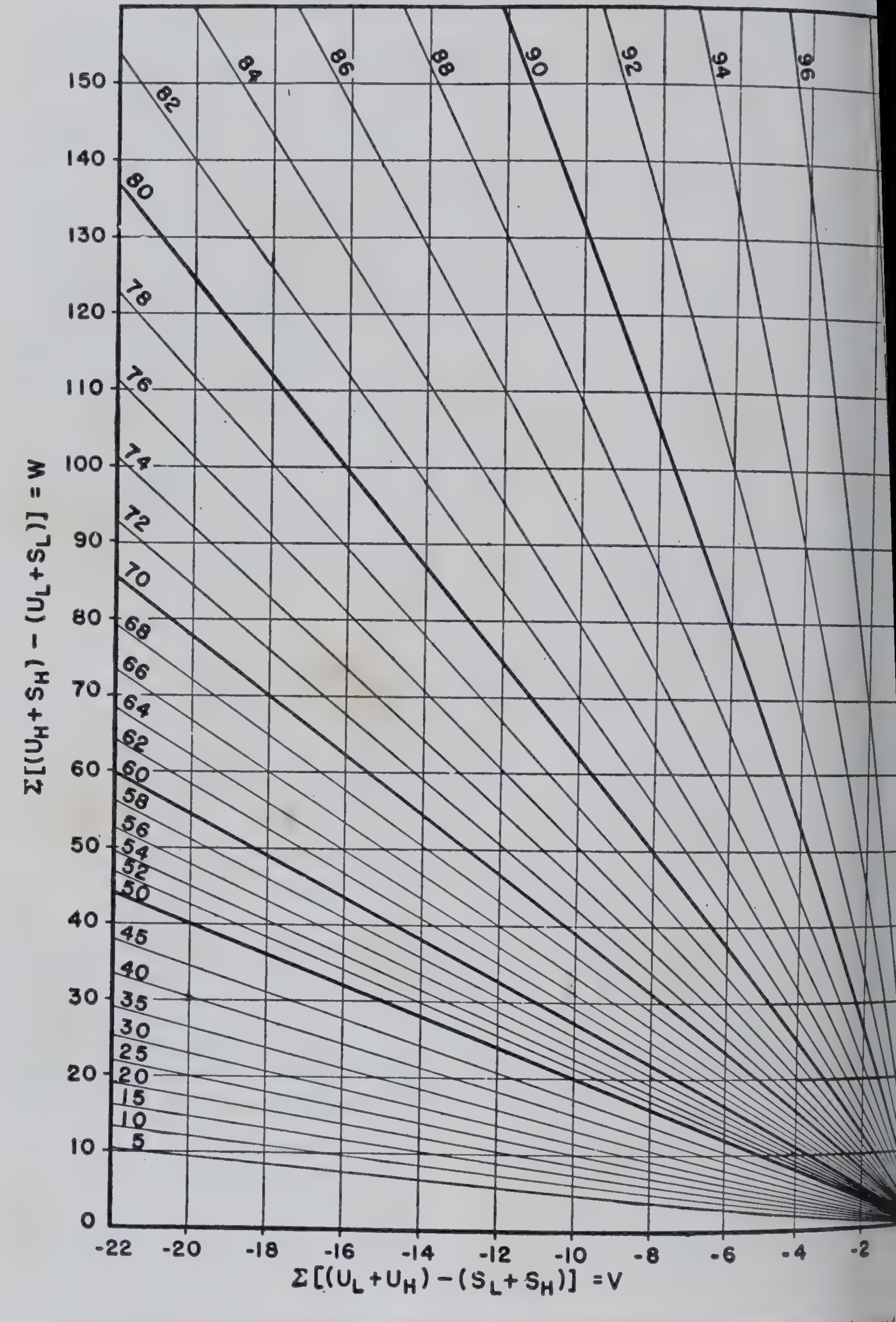
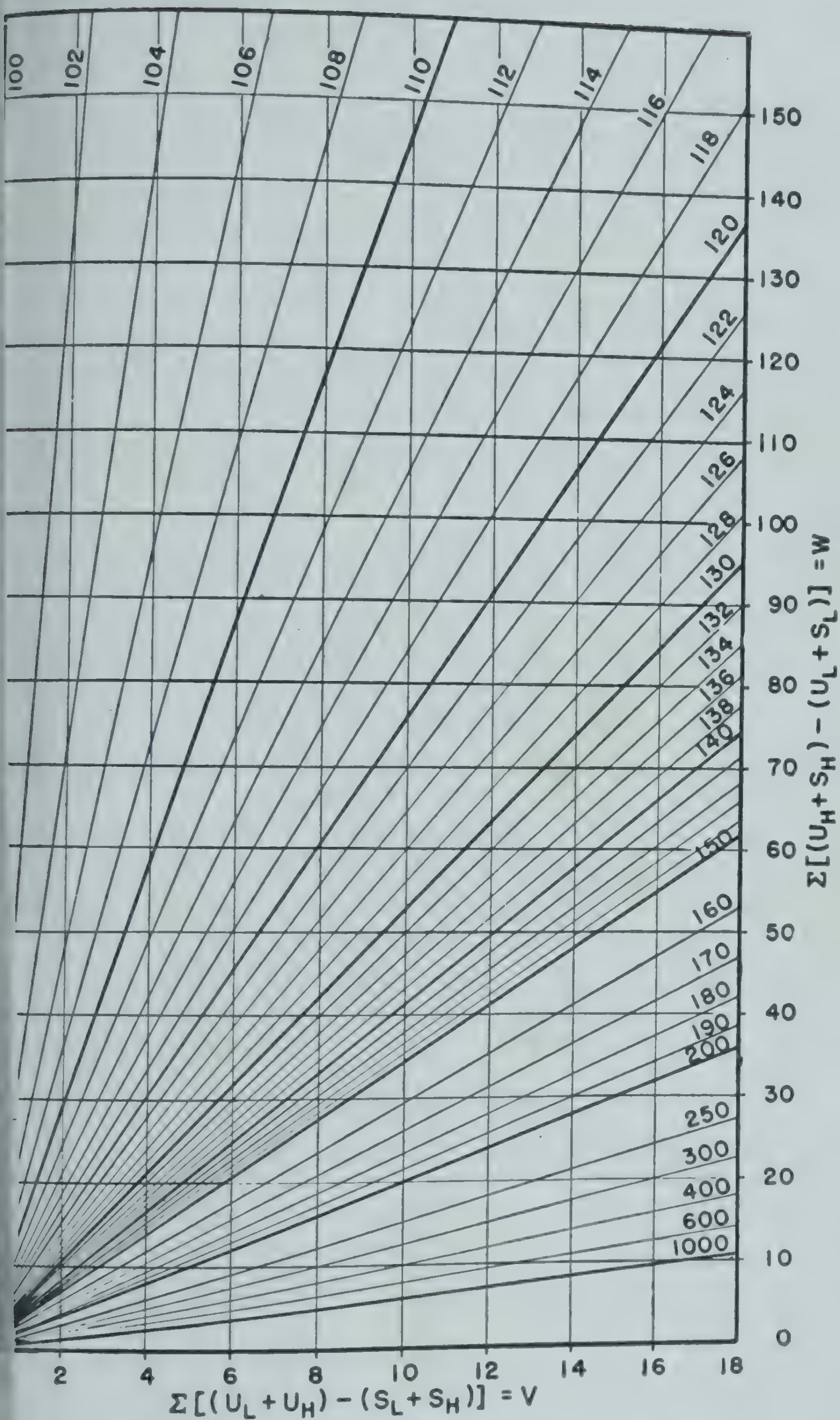


FIG. 134. Penicillin assay chart. (*Courtesy of R. Dugan, Department*



*ucation, and Welfare, Food and Drug Administration.)*

TABLE 220. SOME ASSAY METHODS FOR ANTIBIOTICS

Microbiological Methods

Cylinder-plate Methods:
- Anisomycin (*Saccharomyces cerevisiae,* A.T.C.C. 9763)
- Bacitracin (*Micrococcus flavus,* A.T.C.C. 10240)
- Carbomycin (*Sarcina lutea,* A.T.C.C. 9341)
- Chloramphenicol (*S. lutea,* A.T.C.C. 9341)
- Erythromycin (*S. lutea,* A.T.C.C. 9341)
- Neomycin (*Staphylococcus aureus,* A.T.C.C. 6538P)
- Novobiocin (*Staph. albus,* A.T.C.C. 12228)
- Nystatin (*S. cerevisiae,* A.T.C.C. 9763)
- Penicillin (*Staph. aureus,* A.T.C.C. 6538P) (*S. lutea,* A.T.C.C. 9341)
- Polymyxin (*Brucella bronchiseptica,* A.T.C.C. 4617)
- Streptomycin (*Bacillus subtilis,* A.T.C.C. 6633)
  - Dihydrostreptomycin
- Tetracyclines (*B. cereus* var. *mycoides,* A.T.C.C. 9634)
- Viomycin (*B. subtilis,* A.T.C.C. 6633)

Turbidimetric Methods:
- Dihydrostreptomycin (*Klebsiella pneumoniae,* A.T.C.C. 10031)
- Streptomycin (*Klebsiella pneumoniae,* A.T.C.C. 10031)
- Tetracyclines (*Staph. aureus,* A.T.C.C. 6538P)
- Tyrothricin (*Streptococcus faecalis,* A.T.C.C. 10541)
- Viomycin (*Klebsiella pneumoniae,* A.T.C.C. 10031)

Chemical Methods

Chlortetracycline: Fluorometric method; see *Jour. Am. Pharm. Assoc.* (*Sci.* **38**: 473 (1949).

Chlortetracycline: Acid colorimetric method; see *Jour. Am. Pharm. Assoc.* (*Sci.* **38**: 473 (1949).

Penicillin: Iodometric method.

Streptomycin: Maltol method; see *Jour. Am. Chem. Soc.,* **67**: 2276 (1945); *Biol. Chem.,* **169**: 153 (1947).

Streptomycin and dihydrostreptomycin: Oxidized nitroprusside method; see *Am. Pharm. Assoc.* (*Sci. Ed.*), **41**: 322 (1952).

Tetracyclines: Ferric chloride colorimetric method; see *Jour. Am. Pharm. A* (*Sci. Ed.*), **40**: 241 (1951).

Physicochemical Methods

Chloramphenicol: Ultraviolet spectrophotometric methods; see *Antibiotic* *Chemotherapy,* **1**: 59 (1951).

Dihydrostreptomycin: See *Anal. Chem.,* **23**: 923 (1951).

Fumagillin: See *Jour. Am. Pharm. Assoc.* (*Sci. Ed.*), **43**: 385 (1954).

Oxytetracycline: See Grove, D. C., and W. A. Randall: "Assay Methods of biotics, a Laboratory Manual," Medical Encyclopedia, Inc., New York, 1955.

Tetracycline.

Erythromycin: Spectrophotometric method; see *Antibiotics & Chemotherapy* 1234 (1954).

## SENSITIVITY TESTS

These are tests carried out to determine the relative susceptibility o infectious agent to one or more antibiotics. The objective of the test i discover which antibiotic appears to be the best one to use in the ther

infection. Before any of these tests can be carried out, it is neces-
o isolate in pure culture the primary infecting microbe, which must
ained from the patient before any antibiotic is administered.

re are three main types of sensitivity tests: the broth-serial-dilu-
the agar-dilution, and the agar-diffusion (known also as the
ated-disk") methods. The first of these is considered to be the
ccurate and reliable when properly carried out.

he broth-serial-dilution method, a series of ten 13- by 100-mm.
s placed in a rack. Into each of the first two tubes is placed 0.5 ml.
itable concentration of the antibiotic (usually 100 units or μg. per
Into each of the last nine tubes of the series is placed 0.5 ml. of a
e and sterile broth, such as the penicillin assay broth. The contents
second tube are mixed, and 0.5 ml. is carried to the third tube. The
ts of the third tube are then mixed, and 0.5 ml. of the mixture is
in the fourth tube, and so on through the tenth tube. A 0.5-ml.
of the last tube is discarded. The next step consists of adding to
ibe 1.5 ml. of the same kind of broth medium which contains a
r 1:1,000 or greater dilution of a broth culture of the test organ-
this example, the first tube will contain 25 units per ml. of the
tic; the second tube, 12.5 units; the third tube, 6.25 units; etc. The
re incubated usually at 37°C. for 16 to 18 hr. and then observed
presence or absence of growth. The smallest concentration of
ibiotic which completely prevents growth of the test culture is
l as the minimum inhibitory concentration (MIC). Any number
iotics and many test organisms may be tested by this method. In
determine the relative sensitivity of the test organism to a given
ic, it is necessary to compare the results of a specific test with
tained with other antibiotics. Grove and Randall (1955) have
data in which the response of the microbe is described as sensi-
derately sensitive, and resistant.

late-dilution method is similar in some respects to the broth-
lution method. Dilutions of the antibiotic are prepared, often
initial concentration of 1,000 units per ml. (or μg. per ml.).
etri dishes are marked into 10 approximately equal segments.
of a dilution of the antibiotic and 9 ml. of a suitable agar
such as the penicillin assay agar (at about 48°C.), are added to
te and mixed. After the agar has solidified, each of the 10 seg-
ay be inoculated by streaking or swabbing with a different
For control purposes, bacteria that have known sensitivity,
*Staphylococcus aureus* A.T.C.C. 6538P, may be used to seed
ore of the segments. Control plates, which contain no antibiotic,
seeded with the test organisms. After inoculation the plates are
l at 37°C. for 18 to 24 hr. and then observed. The end point is
entration of antibiotic which completely prevents growth of the
be.

ethod does not give good results with bacteria which give rise to
type or fine discrete colonies or which fail to grow well on agar.

The agar-diffusion, or medicated-disk, method is undoubtedly t
popular and simplest of the sensitivity tests. Plates are poured wit
infusion agar, or other suitable agar medium, with or without the a
of 3 to 5 per cent of sterile defibrinated blood. After solidificati
surface should be dried lightly. The medium is inoculated by st
or smearing the surface with a relatively heavy suspension of t
organism or specimen. The inoculated surface should then be d
that moisture will not be available to leach antibiotic material fr
disks, which are added next. Usually five, six, or more disks, rep
ing low, medium, and high concentrations of the antibiotic, may be
to each plate. Sterile needles, forceps, or other means may be u
place the disks aseptically on the agar. The plates are incubated
to 37°C. for 12 to 16 hr., or longer, and then observed for the pr
of clear zones. No significance is attached to the size of the clear
since there is no direct relationship between the diffusion of the
biotic into agar and its in vivo effectiveness. Results are expressed
for a clear zone of inhibition and 0 (or —) for no zone. Thus, an o
ism which is very sensitive to an antibiotic may show clear zones fo
low, medium, and high concentrations of an antibiotic; a sensitive mi
may show no zone for the low concentration, but clear zones for
medium and high concentrations; a slightly sensitive microbe may
no zones for the low and medium concentrations, but a zone for the
concentration; and a resistant microbe may show no clear zone with
of the concentrations of the antibiotic.

Various modifications of the disk procedure have been reported in
literature. The interested reader should refer to the publications of J
son and Finland (1951), Spaulding and Anderson (1951), and Grove
Randall (1955) for additional details along these lines. The manu
turers of sensitivity disks and antibiotic diagnostic kits have supp
directions regarding the uses of their products.

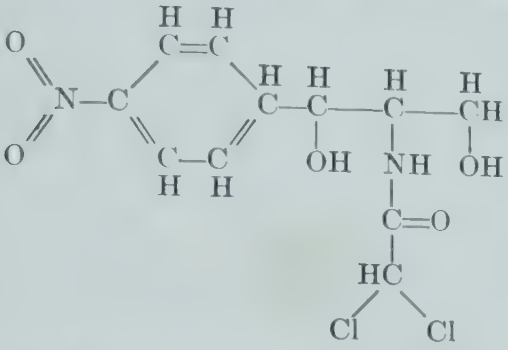
## AMPHOTERICINS

The discovery of two antifungal agents, amphotericins A and B,
duced by *Streptomyces* culture M4575, was reported by Gold et
(1956). The isolation and properties of the crystalline antibiotics
described by Vandeputte et al. (1956). The antibiotics are insolubl
water but are soluble in aqueous alcohols. They are polyenes and amp
teric. Neither one of them is active against bacteria.

Amphotericin B is several times as active toward yeasts and yeast-
fungi as amphotericin A. It is fungistatic in vitro against *Histoplas*
*capsulatum* in amounts of 0.1 to 0.90. μg. per ml. and *Cryptococcus*
*formans* in quantities of 0.03 to 0.16 μg. per ml. (Louria et al. 1957).
decreases the number of *Candida albicans* in the gastrointestinal tr
when administered orally with tetracycline (Halde et al., 1957).
appears to be an antibiotic with considerable promise for the treatm
of mycotic diseases.

## CHLORAMPHENICOL

s antibiotic, first described as Chloromycetin, was discovered by
h and associates (1947). It has the structure shown below:



ramphenicol was first produced commercially by fermentation by
*Streptomyces venezuelae.* It may also be produced by *S. omiyaen-*
ter, after its chemical structure was determined, it was manufac-
y chemical synthesis. Some details of its production on a small
ay be obtained by reference to the report of Smith et al. (1948).
antibiotic is fairly stable in aqueous solutions over a pH range of
for at least 1 day. It is also thermostable in the solid form and
. Like other potent antibiotics, it must be administered with care.
amphenicol is a broad-spectrum antibiotic, which is effective
Gram-positive and Gram-negative bacteria and rickettsiae. It
n found to be particularly effective against *Salmonella typhosa*
tain other *Salmonella* species. It has produced very favorable
n the treatment of typhus fevers, which are caused by rickettsiae.
e but a few of its useful applications.

ward and Wisseman (1957) are the authors of a monograph on
phenicol.

## CYCLOSERINE

serine (trade name, Seromycin) was discovered by Harned and
and described by Harned et al. (1955). It is produced by
*yces orchidaceus.* It appears to be D-4-amino-3-isoxazolidone
$_2O_2$), has a molecular weight of 102, and is amphoteric, water-
nonpyrogenic, and relatively nontoxic. The dry crystals are
room temperature and withstand heating to 100°C. (Marmell
ot, 1956). It has been classified as a broad-spectrum antibiotic
hat low order (Welch et al., 1955). It has shown particular
s an antitubercular agent. Epstein et al. (1956) reported that
efficient antibiotic agent for the treatment of acute and previ-
reated cases of pulmonary tuberculosis, Robitzek and Nenashev
iscussed the effective use of cycloserine with isoniazid in the
of pulmonary tuberculosis.

## ERYTHROMYCIN

This antibiotic, also known as Ilotycin and Erythrocin, is produc
*Streptomyces erythreus* (Waksman) Waksman and Henrici, and wa
described by McGuire and associates (1952). The organism has
described by Waksman (1919), Waksman and Lechevalier (1953)
others.

The antibiotic may be produced in a medium containing soybean
and glucose and extracted with benzene, ethyl acetate, carbon tetra
ride, or other solvents from an alkaline medium.

Erythromycin, a base ($C_{37}H_{67}NO_{13}$), is extremely soluble in ace
acetonitrite, alcohols, chloroform, and ethyl acetate; moderately sol
in amyl acetate, ether, and ethylene dichloride; and soluble in water
concentration of about 2 mg. per ml. It forms salts with acids readily
is stable in the range of pH 6 to 8.

The toxicity of erythromycin is stated to be very low, the $LD_{50}$ for r
by the subcutaneous route being about 1,800 mg. per kg.

Erythromycin is particularly active against Gram-positive bacte
including micrococci, streptococci, pneumococci, and *Corynebacter*
*diphtheriae,* and has shown excellent results in the treatment of infecti
caused by resistant strains of staphylococci (micrococci), particula
those resistant to other antibiotics. It is active also against certain *B*
*cella, Neisseria,* and *Hemophilus* species (Gram-negative bacteria); *D*
*ovania granulomatis; Borrelia recurrentis;* and other microbes.

Erythromycin is usually administered orally as a stearate in the fo
of an oral suspension or tablets. It is readily absorbed from the up
gastrointestinal tract, according to Josselyn and Sylvester (1953). Ery
romycin stearate resists destruction by the gastric juices when adm
istered as an oral suspension (Sylvester and Josselyn, 1953).

The potency of erythromycin is determined by the turbidimet
method, *Streptococcus faecalis* A.T.C.C. 10541 being used as the t
organism.

## FUMAGILLIN

Fumagillin, distributed commercially as Fumidil, is produced
*Aspergillus fumigatus* H-3, a mold similar to but not identical with
*fumigatus* NRRL 171. The antibiotic was isolated in crystalline form
Hanson and Eble (1949) and was noteworthy because it inhibited t
bacteriophage of *Staphylococcus aureus* (strain 209). McCowen et
(1951) discovered that this antibiotic was an extremely potent amoe
cide, particularly against *Endamoeba histolytica* NIH 200. It possess
little antibacterial, antifungal, and antiviral activity. Promising clini
results in the treatment of intestinal amoebiasis have been reported
Killough and associates (1952) and others.

The antibiotic may be produced in a dextrin–corn-steep medium wi
an assay potency of 112 μg. per ml. of beer at 42 hr., according to El
and Hanson (1951).

## GRISEOFULVIN

riseofulvin ($C_{17}H_{17}O_6Cl$) is produced by *Penicillium griseofulvum* ford et al., 1939), *P. nigricans* (*janczewskii*) (Brian et al., 1946, 1949), *P. urticae*. It is slightly soluble in water and too toxic to use sys- tically. It is effective against *Botrytis* species and certain other ls which possess chitin in their walls. It is not effective against fungi cellulose walls (Brian, 1951). It shows promise in enhancing the h of some plants when applied as a spray (refer to page 815).

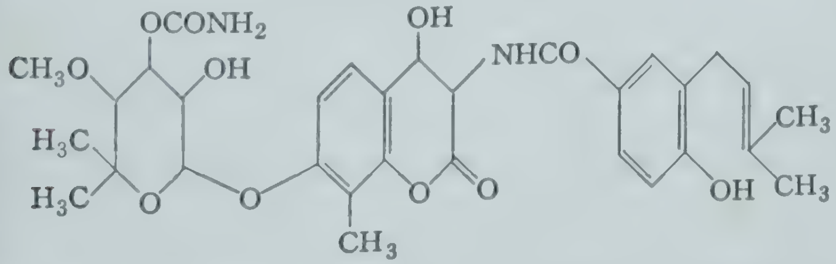
## NEOMYCIN

omycin was discovered by Waksman and Lechevalier (1949). It is nplex produced by *Streptomyces fradiae* and consists of neomycin A, ıd C. The commercial product contains at least 90 per cent of neo- n B. It is a basic substance which forms salts readily with a number ids. It is fairly stable over the pH range of 2 to 9. It is active against ge number of Gram-positive and Gram-negative and acid-fast bac- It is inactive against most yeasts, molds, protozoa, and true viruses. ıse of its neurotoxicity, nephrotoxicity, and ability to produce per- nt deafness, its parenteral applications are limited. The nature, ıtion, isolation, and practical applications of neomycin have been ibed by Waksman (1953). The uses and other pertinent data have discussed by Jawetz (1956).

## NOVOBIOCIN

obiocin is one of the newer commercially produced antibiotics. It iscovered independently by two different laboratories. Smith and ates (1956) reported on its production by *Streptomyces niveus* ). and initially named it streptonivicin. The trade name Albamycin used to identify this product. Wallick and coworkers (1956) ed its production by *S. spheroides* nov. sp. and named it Cathomy- ow a trade name). Welch and Wright (1955) pointed out the com- dentity of streptonivicin and Cathomycin, while Finland (1956) ded that the antibiotics were the same or closely related.

obiocin is a crystalline compound ($C_{31}H_{36}N_2O_{11}$), with the follow- ructural formula elucidated by Hoeksema and associates (1955, , *b*) and Hinman et al. (1956):



dium salt of novobiocin is freely soluble in water and stable at to 9.5 (Frost et al., 1956). It is of relatively low toxicity (Larson et al., 1956). Its production in a synthetic medium has been desc Miller and Walton (1957).

Novobiocin is effective against many of the common pathogen activity has been demonstrated in vitro toward Gram-positive mi streptococci, diplococci, clostridia, and corynebacteria and agai Gram-negative bacteria, such as strains of *Pseudomonas, Pas* *Hemophilus, Proteus,* and *Xanthomonas.* It has shown promisin in the therapy of diseases caused by Gram-positive bacteria, pneumococcal and staphylococcal infections (Cook et al., 1957). studies have indicated that this antibiotic is particularly effective micrococcal infections, especially those resistant to penicillin ( and Lind, 1957). Another feature of this antibiotic is the hig levels obtained by oral administration.

A number of articles concerning the uses of Novobiocin have ap in the "Antibiotics Annual" (1956, 1957).

## NYSTATIN

Nystatin (trade name, Mycostatin) is produced by *Streptomyces* *sei* (Hazen and Brown, 1951). It is an amphoteric, microcrystallin stance with a formula of $C_{46}H_{83}NO_{18}$. It is optically active and ha classified as a tetraene (Pledger and Lechevalier, 1956). It is solu water in the amount of 300 to 600 units per ml. and is stable at thi centration at 5°C. for 1 to 2 weeks. It possesses relatively low toxici is especially effective against many species of yeasts and molds. It limited activity toward bacteria, most protozoa, and filterable v (McLimans et al., 1956). It inhibits the growth of *Penicillium not* *Aspergillus niger,* and *Saccharomyces cerevisiae* in tissue-culture at a concentration of about 31 units per ml. (McLimans et al., 1956). effective against a number of pathogenic microbes, especially *Ca* *albicans,* and *Endamoeba histolytica.* Combination with tetracy oxytetracycline, bacitracin, polymyxin, or fumagillin enhances its bicidal activity (Seneca, 1956).

Resistance studies have been made by Stout and Pagano (1956) studies on clinical uses in treating oral moniliasis have been mad Huang et al. (1956). Clinical experiences with nystatin have bee ported by Eisenberg et al. (1956), Wright et al. (1956), Robinson (1 Seneca (1957), and others.

## OLEANDOMYCIN

Oleandomycin (trade name, Matromycin) is produced by *Str* *myces antibioticus* under submerged aerobic conditions. It was described by Sobin, English, and Celmer (1955). It appears to $C_{37}H_{67}NO_{13}\cdot HCl$ entity, which has been elucidated as a polyhyd polymethyl ketolactone, bound glycosidally with L-oleandrose and d amine (desoxy-sugars). Aqueous solutions of the antibiotic at pH 2 7, and 9 appear to be stable for 24 hr. or more at room temperature. antibiotic appears to possess relatively low toxicity.

andomycin is active chiefly against Gram-positive bacteria, myco-ria, rickettsiae, large viruses, and some protozoa. It has not shown resistance to penicillin, streptomycin, oxytetracycline, chlortetracy-chloramphenicol, polymyxin B, or bacitracin. It is effective in a percentage of cases against antibiotic-resistant clinical isolates of *ylococcus aureus*. Combined with oxytetracycline (Sigmamycin), it s synergistic action toward a number of resistant microbes.

## PENICILLIN

**storical.** Various aspects of the development of the penicillin indus-ave been reviewed by Coghill (1944), Merck and Company (1944), l (1944), Van Winkle and Herwick (1945), Herrell (1946), Fleming (the discoverer), and others.

ming in 1928 observed that a mold contaminant in a culture plate ning staphylococci had caused the dissolution of some of the adja-olonies. He isolated the mold in pure culture; studied the antibac-properties of broth cultures and the crude substance; and concluded, esult of his investigations, that the antibacterial substance, which ned penicillin after the genus of the mold, was relatively nontoxic mals, to leucocytes, and to the conjunctiva of the human eye. He ted that penicillin might have clinical value. The mold isolated by as identified later by Thom, of the U.S. Department of Agriculture, *icillium notatum*.

r Clutterbuck, Lovell, and Raistrick (1932) studied the production covery of penicillin. Information obtained by them has been used sing commercial processes for the extraction of penicillin.

(1935) reported on the antibacterial activity of broth filtrates ntained penicillin.

938, Florey and his associates undertook an extensive study of tics at the Sir William Dunn School of Pathology at Oxford Uni- Penicillin was obtained as a brown powder in fairly good condi-l was used for investigations on animals and human beings. The of these researches were reported by Chain and his associates and by Abraham and his colleagues (1941).

*ime Developments.* In 1941, Florey and Heatley were invited to ted States by the Rockefeller Foundation. Conferences were held iom, principal mycologist of the U.S. Department of Agriculture, rsonnel of the Committee on Medical Research of the Office of ic Research and Development, with the National Research Coun-h the Northern Regional Research Laboratory, and with other As a result of the visits and conferences, drug and chemical man-ng concerns, for example, Merck and Company, E. R. Squibb and harles Pfizer and Company, the Abbott Laboratories, and the p Chemical Company, became interested in the production of n. Meetings of manufacturers were sponsored by Richards, chair-he Committee on Medical Research of the O.S.R.D., for the pur-coordinating the effort, exchanging information, and speeding the nent of the industry.

The rapid and extensive expansion of the penicillin industry on the cooperation, coordination, and vast amount of research ment agencies, of universities, of industrial laboratories, and clinics in this country and England.

**Properties of Penicillin.** Penicillin is active against certa positive bacteria in the presence of blood, pus, and body fluids.

It may be regarded as nontoxic, for very large doses may be tered over a relatively long period of time without injurious eff small percentage of cases, individuals allergic to penicillin a Urticaria, hives, and itching are some of the responses observed tive persons.

According to Welch and his associates (1945), there is partial i of phagocytosis by penicillin.

It is soluble in water. In acetone, amyl acetate, cyclohexane, ethyl acetate, ethyl alcohol, and ether, it is very soluble; in chloroform, and carbon tetrachloride, it is less soluble.

It is unstable under a number of conditions. For example, it i posed by strong acids and alkalies. It is inactivated by oxidizin Contact with certain of the heavy metals, such as copper, silv and mercury, should be avoided, since they tend to destroy p Zinc salts may also inactivate penicillin, according to Eisner a zecanski (1946). The basic structure of penicillin is destroyed by especially methanol, by sulfhydryl compounds, by heat, and by freezing and thawing (Rake and Richardson, 1946).

Penicillin is destroyed by penicillinase, an enzyme secreted by number of bacteria, molds, and yeasts. The aerobic sporeforming and some of the actinomycetes are particularly destructive, W and Foster (1944) have reported. Hence, it is necessary to us media and aseptic methods during the production of penicillin an water or other suitable diluents in the preparation of solutions solid material.

The crystalline salts of penicillin, especially when stored at lo peratures, are stable over a period of several months.

Aqueous solutions of penicillin are unstable and must be store refrigeration.

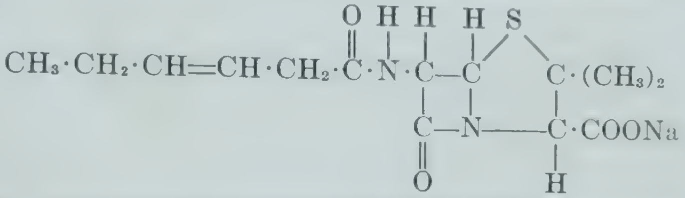
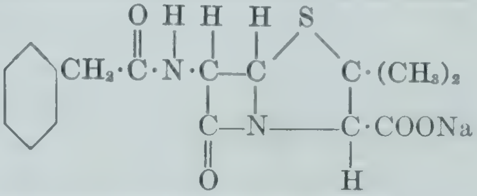
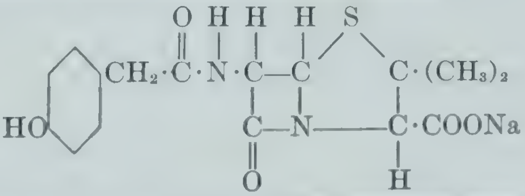
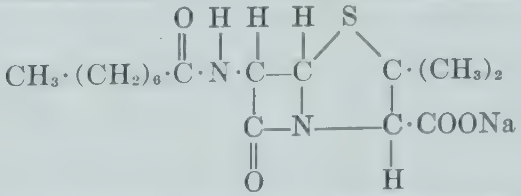
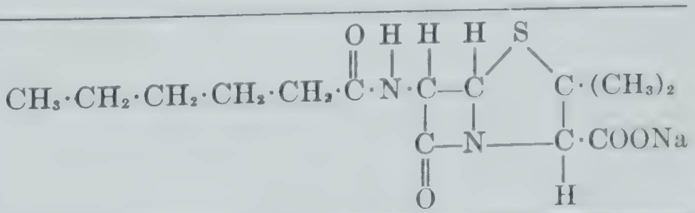
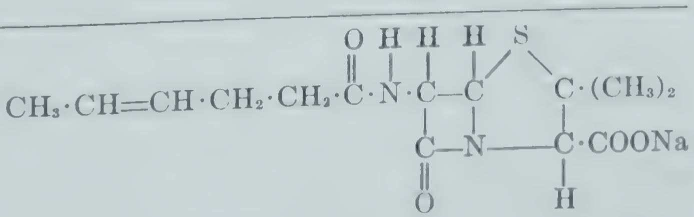
The pH of the aqueous solution containing penicillin has a very tant bearing on the stability of the preparation. Penicillin is mos in the pH range of 6 to 6.5 and reasonably stable over the pH r 5.5 to 7.5. The half lives of penicillins F, G, K, and X are 11, 18.5 11 min., respectively, at pH 2 and 24°C.

Phosphate has a stabilizing influence on crude penicillin at according to Pratt (1947*a*), who found that the rate of loss of varied inversely with the concentration of potassium dihydroge phate present in the culture medium at the beginning. The sta action of the phosphate was independent of the concentrations magnesium sulfate and sodium nitrate. Phenylacetic acid and *p*-h benzoic acid did not modify the stability of crude penicillin when in the medium. Pratt (1947*b*) demonstrated that low concentrat hosphate exerted a stabilizing influence on sterile-water solutions of icillin, which was independent of the buffering effect.

**ypes of Penicillin.** Penicillins are compounds of the general formula $I_{11}O_4SN$—R, in which R represents the radical or group that is different for each type. The structural formulas of the most common natural s (F, G, X, and K) are given in Table 221.

Iost penicillins are mixtures, usually of types F, G, and K, but peni-

TABLE 221. CHEMICAL STRUCTURES OF SOME SODIUM PENICILLINS

| Type | British desig-nation | Structure |
|---|---|---|
| illin F<br>lta$^2$-pentenyl-<br>icillin) | I |  |
| llin G<br>nzylpenicillin) | II |  |
| lin X<br>droxybenzyl-<br>illin) | III |  |
| in K<br>ptylpenicillin) | IV |  |
| o F | Gigantic acid |  |
| n | F type |  |

cillin G is produced in largest quantities and considered to b
practical. Crystalline sodium penicillin G must contain at le
cent of G. Type F penicillin was the predominant type produ
the early years of World War II by surface-culture methods.

*Source.* Penicillins F, G, X, and K are produced by strai
*P. notatum-chrysogenum* group of molds; flavicidin (flavicin
*pergillus flavus;* and dihydro F penicillin (gigantic acid), by
*teus.*

## POLYMYXINS

Polymyxin is produced by strains of *Bacillus polymyxa.* It wa
ered independently by three groups of scientists in 1947: Stans
Benedict and Langlykke, and Ainsworth and associates. There a
ally five different polymyxins, which are designated as polymyx
C, D, and E. These differ somewhat in pharmacologic properties
are effective against certain Gram-negative bacteria. They a
polypeptides which possess molecular weights between 1,050 ar
and which are readily soluble in water. They are stable as ac
They are somewhat neurotoxic and nephrotoxic, but polymyxins
are less toxic than the others. Polymyxin B is manufactured c
cially and is used particularly for systemic infections caused by
*monas aeruginosa* and topically for infections of the eyes, ears, a
caused by Gram-negative bacteria.

Strandskov and Bockelmann (1953) and Haas (1955) have di
the use of polymyxin in the brewery. The former found that pol
was outstanding in its ability to control the infection of brewers
caused by Gram-negative bacteria; the latter observed that polym
inhibited *Pediococcus cerevisiae* development in beer but that it
bactericidal even in relatively high concentrations.

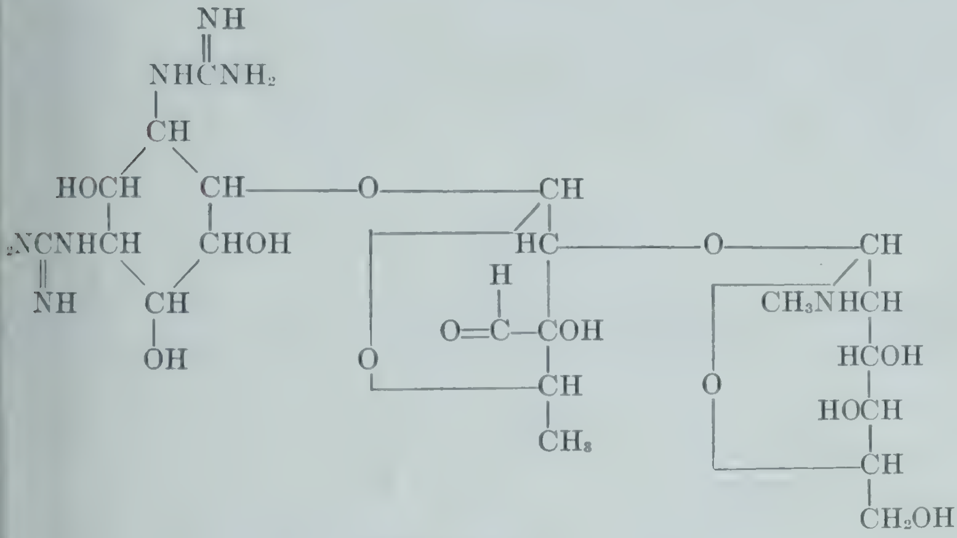
The reader is referred for detailed discussions to the works of
(1956), Pratt and Dufrenoy (1953), Long (1949), and others.

## RISTOCETIN

Ristocetin (trade name, Spontin) is a relatively nontoxic an
produced by *Nocardia lurida* nov. sp. It consists of two similar c
line components, designated as ristocetin A and ristocetin B, whic
tain amino and phenolic groups and sugars and possess molecular
in the range of 2,500 to 4,000 (Grundy et al., 1957*b*). The comp
are amphoteric, soluble, and fairly stable in acidic aqueous solutio
insoluble, in general, in organic solvents (Philip et al., 1957). Ristoc
is reported to be 3 to 4 times as active as ristocetin A in vitro and i
against Gram-positive bacteria and mycobacteria (Grundy et al., 1
Development of resistance, in vitro, to the antibiotic by *Staphylo*
*aureus* has been very slow. Patients with various types of infections
as pneumococcal pneumonia and enterococcal infections, have
rapid improvement (Romansky et al., 1957).

## STREPTOMYCIN

**istorical.** Streptomycin, which was named after the genus of actin- cetes (*Streptomyces*) that produces aerial mycelium and sporulates, discovered in 1943 by Waksman and his associates at the New Jersey cultural Experiment Station, Rutgers University (Schatz et al., ). The finding of this chemotherapeutic agent culminated extensive ys and numerous analyses and tests, all part of a search for an iotic that would be effective against Gram-negative bacteria, bac- statically, bactericidally, in vitro, in vivo, and in the presence of fluids and that would be relatively nontoxic to the cells and tissues e body.



Streptomycin

**es of Organisms against Which Effective.** Streptomycin is par- ly destructive of Gram-negative organisms, for example, *Esch- coli, Aerobacter aerogenes, Pseudomonas aeruginosa, Shigella gal-* , and *Serratia marcescens.* Urinary infections and bacteremias Gram-negative bacteria, meningitis, and other infections caused *nophilus influenzae,* and especially tularemia, are some of the dis- nat may be treated successfully with this antibiotic. Some promise in connection with the treatment of tuberculosis, where strepto- exerts a slow but positive effect against the development of the , which is caused by *Mycobacterium tuberculosis.* Gram-positive rming bacteria, such as *Bacillus subtilis* and *B. cereus,* and certain of *Staphylococcus aureus,* are susceptible to the influence of this ic.

**erties of Streptomycin.** Streptomycin is soluble in water and e in acetone, chloroform, and ether. It is readily adsorbed on l from alkaline solutions and may be eluted with dilute acid . Streptomycin is relatively stable. According to Regna, , and Solomons (1946), there was no loss of potency in commer- ples that were stored at room temperature for 12 months and that

ined less than 1 per cent of moisture. They also reported
no loss in potency over a period of 60 days in aqueous
tomycin at a pH of 3 to 7 and stored at or below 28°C. H
per cent reduction in activity resulted from storage of a str
solution at a pH of 5.5 and at a temperature of 95°C. for 37 h
ing to Waksman and Schatz, less than 50 per cent of the a
streptomycin was destroyed by heating it at 100°C. for 10 mi
and Nielsen reported that solutions containing 100 and 1,000 un
of streptomycin at pH 6, 7, and 8 and stored at 10°C. were
3 months.

Strong alkaline solutions are destructive to streptomycin, t
being very rapid when the solutions are boiled.

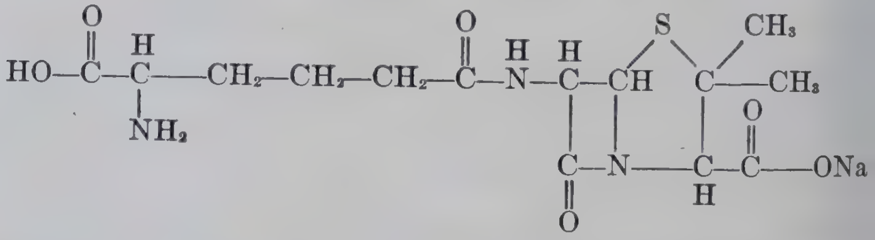
Streptomycin is highly resistant to the action of enzymes a
biological agents.

Crude and highly concentrated solid preparations are hygrosc

**Production.** The commercial production of streptomycin h
described by Silcox (1946), Porter (1946, 1947), Kirkpatrick
and others.

## SYNNEMATIN B

This antibiotic is produced by *Cephalosporium salmosynnemat*
the sodium salt has the following structural formula (Clark et al.,



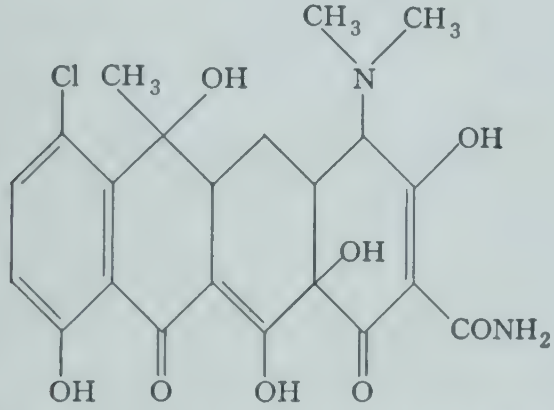
It will be noted that it contains the penicillin nucleus and an ami
moiety. Its discovery was reported by Gottshall and associates i
It is identical with cephalosporin N (Abraham et al., 1955), an an
discovered independently by Burton and Abraham (1951). Its p
tion with ion-exchange resins has been described by Clark and ass
(1957). Penicillinase degrades its antibacterial action. The an
is inactivated by a pH less than 4 or greater than 9 (Abraham
1953). Hwang and Primack (1957), in acute and subacute studie
mice, rats, rabbits, and dogs, observed that it was extremely no
Intramuscular administration of aqueous solutions and suspension
were well absorbed; however, a number of pharmaceutic prepa
administered orally were absorbed in a poor manner (Sylvester
1957). Synnematin B is effective against some Gram-positive as
Gram-negative bacteria. According to Hobby and associates, it is
active in vitro and in vivo against various species of *Salmonella.*

The biochemistry of the cephalosporins has been discussed by Ab
(1957).

## THE TETRACYCLINES

his group of compounds includes chlortetracycline, oxytetracycline, tetracycline, which are classified as broad-spectrum antibiotics. In ral, the antibiotics of this group are active against Gram-positive Gram-negative bacteria and species of *Rickettsia* and possess rela- ly low toxicity. They are widely used for combating infections in an beings and animals, including amoebiasis, diarrheas, shigellosis, other disease conditions. In addition chlortetracycline and oxytetra- ine are used in animal feeding, for the preservation of foods, and for r purposes.

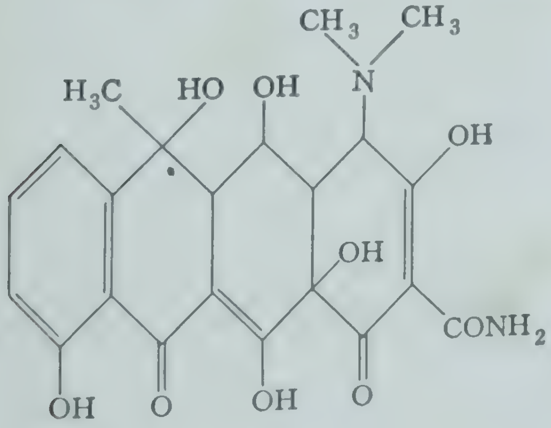
**lortetracycline.** This antibiotic, known also as Aureomycin and mycin, was discovered by Duggar (1948) and has the following struc- l formula:



roduced by *Streptomyces aureofaciens,* an actinomycete which has lescribed by Duggar (1948).

ailed accounts of the properties and uses of this antibiotic have been ted by Duggar et al. (1948), Lepper (1957), and others. Numerous ations have appeared in the literature concerning the clinical appli- s of this outstanding drug. Reference to applications in the agricul- ield and food industry appears in a later section of this book.

**tetracycline.** This antibiotic, also known as Terramycin, was dis- d by Finlay et al. (1950). The structural formula follows:



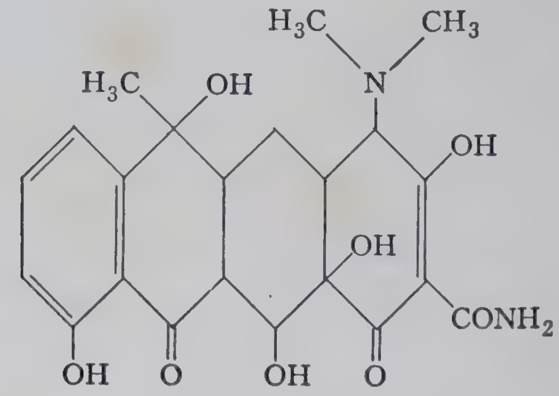
Oxytetracycline is produced by *S. rimosus*. It is amphoteric a crystalline hydrochloride and sodium salt. It is fairly stable i solutions at about pH 2 to 5 at room temperature. It posse degree of toxicity.

A considerable amount of information concerning *S. rimosus* a in producing the antibiotic may be found in U.S. Patent According to Sobin, Finlay, and Kane (1950), carbon may b by sugars, starch, or glycerol; organic nitrogen, by soybean me gluten, cottonseed meal, lactalbumin, the enzymatic digest of tryptone; growth substances, by distillers' solubles or yeast ext mineral requirements, through the use of such salts as sodium potassium phosphate, magnesium sulfate, and sodium nitrate. carbonate may be used for buffering purposes. One fermentation cited by Sobin et al. contained 3 per cent soybean meal, 0.5 cornstarch, 0.1 per cent N-Z-Amine B (enzymatic digest of ca per cent $NaNO_3$, 0.5 per cent $CaCO_3$, and 0.4 per cent vege These ingredients are dissolved in tap water, and the pH is adju The medium is sterilized for 1 hr. at 121°C. and cooled to 28°C. ing. Production is carried out by the submerged process with and aeration at the rate of 0.5 to 2 volumes of air per volume of per minute. The temperature may be varied from 24 to 30°C., d on the conditions.

Data concerning the development of the process for making o cycline have been presented by Reeves (1952), while Regna et al have reported on the isolation of this drug from production m titers of about 1,000 μg. per ml.

Musselman (1957) is the author of a monograph concerning o cycline.

**Tetracycline.** This antibiotic may be produced by chemical s or by fermentation (Putnam et al., 1953). It has the following st formula:



Details concerning the development, chemistry, pharmacology peutic effects, and clinical usefulness of this antibiotic have be cussed in a monograph by Dowling (1956).

The manufacture of this antibiotic has been described by I (1957), who has presented a flow sheet for the process.

## VANCOMYCIN

This antibiotic (trade name, Vancocin) was isolated from strains of *eptomyces orientalis* nov. sp. by McCormick et al. (1956), who de- ibed a method for its production by a submerged-culture process, puri- tion, chemical properties, and biologic activity. Vancomycin hydro- ride is a white solid, freely soluble in water. It is relatively stable in l media. It is a complex amphoteric substance with a molecular ght of about 3,300. It is effective against Gram-positive bacteria, cially cocci. It appears to be active against multiplying bacteria only aci et al., 1957). The development of resistance to vancomycin several strains of *Staphylococcus aureus* is slower than with many r antibiotics (Ziegler et al., 1956). No cross resistance with other biotics has been observed with staphylococci (Kirby and Divelbiss, ). Pharmacological and toxicological studies have indicated that omycin possesses a low order of toxicity toward various species of ratory animals (Lee et al., 1957).

## ANTIBIOTICS AS FOOD PRESERVATIVES

ntibiotics are being used commercially to extend the storage life of hable foods, particularly poultry, fish, and meat. Advantages of the od include the saving of foods, increased availability of foods in areas of the world where refrigeration is scarce or lacking, and oved quality of food in some instances.

tibiotics should be used in foods with great care, in order to protect ealth of the consumers. Health authorities are concerned with pre- ng the addition of materials to foods which may produce toxicity, e sensitivity to antibiotics, or bring about the emergence of strains hogens resistant to these drugs.

ler the provisions of Section 408 of the Federal Food, Drug, and etic Act, safe tolerances for antibiotics in or on agricultural com- ies may be established when there is satisfactory evidence available ir usefulness and of the safety of their residues (Durbin, 1956).

ording to Durbin, antibiotics may be used in foods in the United provided that they meet certain requirements. They may be used led that no residues remain in the foods. Likewise, they may be o that residues remain in uncooked foods, provided that these foods ways cooked, that cooking always destroys all of the antibiotic, at the residues in the *uncooked* foods are within official tolerance.

ain antibiotics are used commercially to extend the storage life of ble foods. The more important ones are chlortetracycline (Aureo- , oxytetracycline (Terramycin), and nisin. The tetracyclines have ed to preserve poultry, beef, and fish. Chlortetracycline and oxy- cline may be used in the United States for preserving poultry, pro- hat the tolerance of 7 parts per 1,000,000 of antibiotic residue in he uncooked poultry is not exceeded in any part of the poultry.

Antibiotics may be used in Canada for preserving fish and poultr
tetracyclines are used in Brazil to preserve fish, poultry, and r
Nisin is used in Great Britain, France, and elsewhere to contro
fermentation during the making of certain types of cheeses and t
the development of *Clostridium* species.

**Preservation of Poultry.** Studies relating to the preservation
try with antibiotics have been reported by Spencer et al. (1954),
and Stadelman (1955), Kohler et al. (1955), Miller (1956), Kohl
(1957), Windlan (1957), and others.

Ziegler and Stadelman showed that halves of chicken fryers im
for 10 min. in aqueous solutions containing 10, 20, or 40 p.p.m. of
mycin and stored at 2.2°C. remained fresh several days longer tha
trols not treated with the antibiotic.

Kohler and associates (1955) demonstrated that poultry immer
2 hr. in a cooling tank containing 10 p.p.m. of chlortetracycline (
mycin) was in a satisfactory condition after storage at 3°C. for 3
a significantly longer time than was true of untreated controls.

The commercial process is briefly about as follows: The poultry
pared by killing, dressing, eviscerating, and weighing. The ant
preparation, which contains 10 per cent, or 20 per cent, of anti
citric acid, and other edible ingredients, is usually dissolved in a
quantity of potable water (free of chlorine) and added to the slush
cooling tank in such concentration as to yield 10 p.p.m. of the anti
in the reaction mixture. The antibiotic is thoroughly mixed wit
slush (ice and water). The poultry is added in such fashion tha
entirely immersed in the antibiotic-containing solution and then co
with crushed ice. After 0.5 to 2 hr. (preferably about 2 hr.), the p
is removed, drained, and packed in ice in the usual manner. A mod
tion of the foregoing procedure is necessary if the installation
in-plant chlorination. The tanks used for antibiotic treatment shou
lined with stainless steel or glass.

**Preservation of Fish.** Tarr, Southcott, and Bissett (1950);
Boyd, and Bissett (1954); Farber (1954); Boyd and Tarr (1956);
(1956); Tomiyama et al. (1956); Firman et al. (1956); Klein
(1956); and others have studied the effects of antibiotics on fish.

A number of antibiotics have been used in experimental studies.
Aureomycin, Terramycin, and chloramphenicol were effective unde
conditions of use.

Various methods have been used to treat the fish with antibiotics.
et al. (1954) reported that the spoilage of whole, eviscerated fish
markedly retarded (1) by using ices containing 1 to 4 p.p.m. of c
tetracycline, (2) by holding them 6 days at −1°C. in sea water which
tained 2 p.p.m. of the antibiotic, or (3) by immersing them for 1
before icing, in solutions containing 50 to 100 p.p.m. of the antib
Farber (1954) observed that dipping fish fillets (ling cod, sole, black
and sable fish) in water containing 2 p.p.m. of Aureomycin or Terran
prolonged their keeping quality. Tomiyama et al. (1956) found tha
storage life of round herring was prolonged remarkably (1) by sto

le on the boat, in sea water containing ice and 10 p.p.m. of Aureo-in; (2) by storage, after landing, in an ice containing 5 p.p.m. of eomycin; or (3) by a combination of these methods.

irman and collaborators (1956) reported that the fresh quality of fish, as sea bass, weak fish, croaker, scrod, butterfish, salmon, halibut, porgy, may be prolonged successfully by the application of chlor-acycline in the form of an ice, dip, or freezing brine. The ice contained p.m. of the antibiotic and was prepared in the manner outlined by am et al. (1955). For dipping purposes, the fish were usually sub-ged for 10 or 20 min. in fresh or sea water which contained 10 p.p.m. ie antibiotic, for 30 min. in sea water which contained 5 p.p.m. of ntibiotic, or in 22 per cent freezing brine which contained 10 p.p.m. e antibiotic.

llortetracycline has been used to aid in the preservation of whales nmals). For a 50-ton whale, 55 g. of the antibiotic, corresponding to t 1 μg. per g., was used. A solution of the antibiotic is introduced igh the air line, which is used for inflating the whales before they owed. A charge of 25 g. of the antibiotic was placed in a linen bag in omb head of the harpoon in one case.

yd and Tarr (1956) have studied the effect of chlortetracycline and ge temperatures on the quality of shucked oysters.

**eservation of Fresh Meat.** Goldberg and associates (1953), Weiser (1953, 1954), Deatherage (1956), and other scientists have studied ffects of antibiotics in preserving fresh meat. Goldberg et al. found Chloromycetin, Terramycin, and Aureomycin at concentrations of m. increased the storage life of ground beef and were highly active d pure cultures of bacteria isolated from beef tissues. Penicillin, acin, and streptomycin were ineffective in delaying spoilage of the es. Weiser and collaborators (1953, 1954) found that the infusion inds and whole animals with saline containing Aureomycin may it deep spoilage when refrigeration is delayed. It was observed that ef infused with the antibiotic presented a slightly altered appear-however, the eating quality could not be distinguished from that of ted beef.

ole animals may be infused with antibiotics in a number of differ-ys. In one method, described by Deatherage (1956), the animal is d, bled through the jugular vein and/or carotid artery, and placed back. The carotid arteries are exposed; one is clamped, and the is used to introduce an infusion of the antibiotic. The amount of te used is usually one-third the blood volume. It takes 3 to 4 min. ct the material. Afterward the animal is dressed in the customary r. The infusate may contain 55 p.p.m. of the antibiotic (0.75 g. per of physiological saline), according to Weiser et al. (1953). Gen-the amount of material infused may amount to 10 per cent of the of the animal. On the basis of an animal weighing 1,000 lb., this correspond to the addition of 100 lb. of infusate, or 2.5 g. of anti-which is equivalent to roughly 5 p.p.m. The carcass, after skin-ay be sprayed with the antibiotic solution to reduce surface spoil-

age. The method works reasonably well with cattle and sheep b
difficult to perform with swine.

Another method involves intraperitoneal injection of the
solution 1 to 2 hr. before slaughter. Problems include those per
the handling of untamed animals, the availability of only a s
for injection in cattle (because of the rumen) and in fat animals
of the kidneys and surrounding fat), possible precipitation of
solutions of the tetracyclines in the peritoneal cavity, and other di

Antibiotics are being used to preserve red beef in several
particularly in areas where refrigeration is absent or scarce.

**Preservative Action of Nisin.** The antibiotic nisin is prod
certain strains of *Streptococcus lactis*. It is of interest becaus
ability to retard the development of clostridia in food prod
inhibits the clostridia which are occasionally a cause of gas pr
in cheeses, such as Gruyère. The use of dry nisin preparation
pressing the growth of food-poisoning bacteria, such as *Clostridiu*
*linum,* in bread, cheese, meat broths, meat pies, and sausages h
patented by Hawley (1955). Nisin may be incorporated in the fo
liquid or solid preparation, or a nisin-producing strain of *S. lactis*
inoculated into the food. Some references concerning nisin will b
in Table 222.

**Other Uses of Antibiotics.** Table 222 contains information
to studies carried out on some food substances with various antibi

In closing this rather brief discussion on the use of antibiotics
preservatives, the author wishes to emphasize the importance o
antibiotics intelligently and in accordance with existing regulat
governmental and health authorities. Carefully used, antibiotics
very valuable; improperly used, they may constitute a menace
food industry and to the health of the consuming individual.

## ANTIBIOTICS AND PLANT DISEASES

A considerable amount of research and study has been carried
plant pathologists since 1950 to determine the value of antibiot
controlling plant diseases of bacterial and fungal origin. Of th
biotics studied, streptomycin appears to be the most important.
nation of Terramycin (oxytetracycline) with streptomycin has en
the latter's value (Visor et al., 1955). Actidione (cycloheximid
given satisfactory results against some plant diseases caused by
Griseofulvin, anisomycin, and other antibiotics offer considerable
ise in protecting certain plants and eradicating disease.

Table 223 lists some of the more important plant diseases whic
been treated successfully with streptomycin alone or combine
Terramycin.

Streptomycin appears to be quite stable in plant tissue (Goodm
Johnston, 1957). It is apparently not degraded or metabolized a
ably in plant tissue, nor is it inactivated by cooking for short
of time.

| | | Reference |
|---|---|---|
| Canned foods (spores, etc.) | Subtilin, etc. | Andersen and Michener: *Food Technol.*, **4**: 188 (1950).<br>Cameron and Bohrer: *Food Technol.*, **5**: 340 (1951).<br>Campbell and O'Brien: *Food Technol.*, **9**: 461 (1955).<br>Denny et al.: *Natl. Canners' Assoc., Research Rept.*, **6**: 53 (1953).<br>LeBlanc et al.: *Food Technol.*, **7**: 181 (1953).<br>Lewis et al.: *Jour. Agr. Food Chem.*, **2**: 298 (1954).<br>Michener: *Appl. Microbiol.*, **1**: 215 (1953).<br>Williams and Campbell, Jr.: *Food Research*, **16**: 347 (1951).<br>Wrenshall: *Food in Canada*, **13**: 25 (November, 1953). |
| Cheeses | Nisin | Toravill, Mattick, and Hirsch: British Patent 713,251, Mar. 7, 1952.<br>McClintock et al.: *Jour. Dairy Research*, **19**: 187 (1952).<br>Kooy and Pette: *Neth. Milk Dairy Jour.*, **4**: 302, 317 (October–December, 1952).<br>Berridge: *Chemistry & Industry*, 1953, p. 1158.<br>Hawley: Canadian Patent 511,616, Apr. 5, 1955. |
| Custard fillings: | | |
| *Staphylococcus aureus* | Subtilin | Godkin and Catheart: *Food Technol.*, **6**: 224 (1952). |
| | Subtilin + Terramycin | |
| *Salmonella* | Subtilin + Terramycin | —— and ——: *Food Technol.*, **7**: 282 (1953). |
| Milk | Terramycin, Aureomycin | Greene and Bell: *Sci. Agr.*, **32**: 619 (1952). |
| | Penicillin, streptomycin, chloramphenicol, patulin, Terramycin, Aureomycin | Hashida: *Fermentation Technol. Japan*, **31**: 15, 112 (1953). |
| | Aureomycin and others | Shiveler and Weiser: *Jour. Milk and Food Technol.*, **16**: 125 (1953). |
| Spinach | Streptomycin | Smith: *Phytopathology*, **42**: 475 (1952). |

TABLE 223. SOME DATA ON SOME PLANT DISEASES CONTROLLED THROUGH THE USE OF ANTIBIOTICS

| Plant disease | Causative agent | Reference |
|---|---|---|
| Bacterial spot of tomato and pepper.. | *Xanthomonas vesicatoria* | Coe: *Plant Disease Reptr.,* **39**: 215 (1955).<br>Conover: *Plant Disease Reptr.,* **38**: 405 (1954); **39**: 611 (1955).<br>Cox: *Plant Disease Reptr.,* **39**: 484, 616 (1955). |
| Bacterial wilt of chrysanthemums.. | *Erwinia chrysanthemi* | Robinson, Starkey, and Davidson: *Phytopathology,* **44**: 646 (1954). |
| Blackleg and seed-piece decay of potatoes | *E. atroseptica*<br>*Pseudomonas fluorescens* | Bonde: *Am. Potato Jour.,* **30**: 143 (1953).<br>———: *Plant Disease Reptr.,* **39**: 120 (1955). |
| Fire blight of apples and pears....... | *E. amylovora* | Ark: *Plant Disease Reptr.,* **37**: 404 (1953).<br>——— and Scott: *Phytopathology,* **44**: 481 (1954).<br>Dunegan et al.: *Plant Disease Reptr.,* **38**: 666 (1954).<br>Goodman: *Am. Fruit Grower,* **73**: 7 (1953).<br>———: *Proc. Am. Soc. Hort. Sci.,* 1954, p. 186.<br>———: *Plant Disease Reptr.,* **38**: 874 (1954).<br>Kirby: *Plant Disease Reptr.,* **38**: 432 (1954).<br>Mills: *Plant Disease Reptr.,* **39**: 206 (1955).<br>Young and Winter: *Plant Disease Reptr.,* **37**: 463 (1953). |
| Halo blight of beans ............... | *Ps. phaseolicola* | Mitchell et al.: *Science,* **115**: 114 (1952).<br>———: *Phytopathology,* **44**: 25 (1954).<br>Zaumeyer: *Jour. Agr. Food Chem.,* **3**(2): 112 (1955).<br>——— et al.: *Am. Vegetable Grower,* **1**: 5 (1953).<br>———: *Phytopathology,* **43**: 407 (1953). |
| Tobacco blue mold ................. | *Peronospora tabacina* | Grosso: *Plant Disease Reptr.,* **38**: 333 (1954). |
| Tobacco wildfire .................. | *Ps. tabaci* | Beach and Engle: *Plant Disease Reptr.,* **39**: 15 (1955).<br>Heggested and Clayton: *Plant Disease Reptr.,* **38**: 661 (1954) |

ctidione has given good results in the treatment of cherry leaf spot, dollar spot of turf, and certain other diseases of fungal origin. The biotic is somewhat phytotoxic even in low concentrations and thus be used with special care.

umeyer (1957), on the basis of greenhouse tests, reported that the lery mildew of beans, which is caused by *Erysiphe polygoni*, may be olled effectively with sprays of anisomycin and griseofulvin. Sprays aining 50 p.p.m. of anisomycin, or 100 p.p.m. of griseofulvin, pro- d the beans from powdery mildew. A concentration of 200 p.p.m. iseofulvin, or of 50 p.p.m. of anisomycin, was required to eradicate ungus. Fungichromin, nystatin, oligomycin, rimocidin, and strepto- n were ineffective in preventing powdery mildew.

isomycin protects beans from rust caused by *Uromyces phaseoli a* and lima beans from downy mildew caused by *Phytophthora oli* (Zaumeyer and Wester, 1956). Griseofulvin likewise protects from rust.

seofulvin may be useful in controlling the blight and dieback of tions caused by *Alternaria dianthi* and *Fusarium culmorum*, respec- (Rhodes, 1955). It has other potential uses (Brian, 1952; Stubbs, Zaumeyer, 1957).

ays may be prepared to contain 1 to 500 p.p.m. of the antibiotic, ding on the intended use. The number of individual applications ie spray schedule depend on the disease or crop. Trees, plants, seeds, gs, and seedbeds may be treated with the antibiotics.

ibiotics useful in promoting plant health may be absorbed by the nd cut surfaces, may penetrate the epidermal layers in the aerial s, and may be translocated in the vascular system. Wetting such as Tween 20 (0.005 per cent) (Zaumeyer, 1957) and Triton (Ark, 1954), glycerol, and other substances have been used to e the effectiveness of spray materials.

ket preparations of antibiotics include Agri-mycin, which contains ent streptomycin in the sulfate form and 1.5 per cent Terramycin; ept, which contains 37 per cent commercial streptomycin in the sul- m; Acco streptomycin, which contains 45 per cent streptomycin; ycin, which contains 20 per cent commercial streptomycin as the in liquid form; and actidione (Zaumeyer, 1956).

neyer (1955, 1956) has reviewed the subject of improving plant through the use of antibiotics, and the interested reader is referred ublications and those of others for details.

## ANTIBIOTICS AND ANIMAL NUTRITION

iotics are used in the feeding of chickens, turkeys, swine, young lambs, and other animals. The antibiotics which have been used idely include penicillin, chlortetracycline, and oxytetracycline. nycin, dihydrostreptomycin, bacitracin, and chloramphenicol have ed to a much smaller extent. Penicillin appears to be the anti- first choice for feeding chickens and turkeys; however, chlor-

TABLE 224. SOME ANTIFUNGAL ANTIBIOTICS

| Antibiotic | Produced by | Effective against * | Reference † |
|---|---|---|---|
| Actinone ........... | *Streptomyces* sp. (closely related to *S. antibioticus*) | *Saccharomyces* and *Trichophyton* spp. | Ikeda et al.: *Antibiotics* Japan, **3**: 724 (1950). |
| Amphotericins ...... | *Streptomyces* M4575 | *Histoplasma capsulatum* and other pathogenic fungi | Gold et al.: "Antibiotics Annual 1956–1957." Medical Encyclopedia, Inc., New York, 1957, p. 579. |
| Antimycin A ........ | *Streptomyces* sp. | Yeasts and filamentous fungi | Leben and Keitt: *Phytopathology,* **38**: 899 (1948). |
| Antimycoin ......... | *S. aureus* | Fungi | Raubischek et al: *Antibiotics & Chemotherapy,* **2**: 179 (1952). |
| Ascosin ............ | *S. canescus* | Chiefly against yeasts, especially *S. cerevisiae* | Hickey et al.: *Antibiotics & Chemotherapy,* **2**: 472 (1952). |
| Cacaomycetin ...... | *S. cacaoi* | *Aspergillus niger* and certain ascomycetes and phycomycetes | Wakaki et al.: *Antibiotics* Japan, **5**: 24 (1952). |
| Candicidin ......... | *S. griseus* and other species | Yeasts, certain filamentous molds, and *Candida albicans* | Lechevalier et al.: *Mycologia,* **45**: 155 (1953). |
| Chromin ........... | *S. antibioticus* | Yeasts and filamentous fungi | Wakaki et al.: *Antibiotics* Japan, **5**: 677 (1952). |

* Special reference is made to yeasts and filamentous fungi. Activity against bacteria and other microbes has been demonstrated by
of the antibiotics in addition to the antifungal properties.

| | | | |
|---|---|---|---|
| | | | and Hazen: "Antibiotics Annual 1955–1956," Medical Encyclopedia, Inc., New York, 1956, p. 245. |
| Eulicin ............ | *Streptomyces* sp. (related to *S. parvus*) | Various pathogenic fungi and *A. niger* | Charney et al.: "Antibiotics Annual 1955–1956," Medical Encyclopedia, Inc., New York, 1956, p. 228.<br>West et al.: "Antibiotics Annual 1955–1956," Medical Encyclopedia, Inc., New York, 1956, p. 231. |
| Fradicin ........... | *S. fradiae* | Many fungi | Swart et al.: *Proc. Soc. Exptl. Biol. Med.*, **73**: 376 (1950). |
| Griseofulvin ........ | *Penicillium griseofulvum* | Certain phytopathogenic molds and other fungi | Brian: *Botan. Rev.*, **17**: 357 (1951). |
| Mycelin ............ | *S. roseoflavus* | Some filamentous fungi | Aiso et al.: *Antibiotics* Japan, **5**: 217 (1952). |
| Mycomycin ......... | *Nocardia acidophilus* | Some fungi | Celmer and Solomons: *Jour. Am. Chem. Soc.*, **74**: 1870 (1952). |
| Mycothricin ........ | *S. lavendulae* | Yeasts and filamentous fungi | Schaffner et al.: *Proc. 3d Intern. Congr. Biochem.*, Brussels, 1955. |
| Nystatin ............ | *S. noursei* | Many species of yeasts and molds | Hazen and Brown: *Science*, **112**: 423 (1950). |
| Rimocidin .......... | *S. rimosus* | *C. albicans* and other pathogenic fungi | Davisson et al.: *Antibiotics & Chemotherapy*, **1**: 289 (1951). |
| Rotaventin ......... | *S. reticuli* | Certain yeasts and molds | Hosoya et al.: *Antibiotics* Japan, **5**: 451 (1952). |
| Thiolutin .......... | *S. albus* and *S. celluloflavus* | Certain pathogenic fungi | Seneca et al.: *Antibiotics & Chemotherapy*, **2**: 357 (1952). |

tetracycline and oxytetracycline are in close second place. C
cycline and oxytetracycline seem to be the antibiotics of choic
ing young calves.

The level of antibiotic fed depends on the animal and the r
ordinarily 50 to 200 to 1,000 g. of antibiotic is included with 1 t
and 25 to 250 p.p.m. of the antibiotic in the drinking water
latter is used as the vehicle.

Antibiotics seem to benefit nutrition in a general way and t
growth. The rate of gain in weight is marked in some cases,
when the animal may have been suffering from gastroenteri
low-quality rations. Antibiotics appear to stimulate the feed
poultry and reduce the mortality rate. The growth effect of anti
chickens and swine reared in new quarters is lacking or very low

The reader is referred to the works of Jukes (1955), the Proce
the First International Conference on the Use of Antibiotics in
ture (1956), and other sources for additional information.

## OTHER USES OF ANTIBIOTICS

Antibiotics are used for a number of other purposes on a limi
They are useful as special constituents of culture media for inhib
development of undesirable types of microorganisms, as, for exa
isolation techniques (Dunn, 1955) and in the enumeration of ba
bakers' yeast (Green and Gray, 1951).

Antibiotics have been studied for their possible use as contan
control agents in special fermentations (Strandskov and Boc
1953; Day et al., 1954; Dunn, 1955).

## ANTIFUNGAL ANTIBIOTICS

Some data relating to antifungal antibiotics are presented in T

### Some References concerning the Antibiotics

#### AMPHOTERICINS

Gold, W., H. A. Stout, J. F. Pagano, and R. Donovick: Amphotericins A an
fungal Antibiotics Produced by a Streptomycete. I. In Vitro Studi
biotics Annual 1955–1956," Medical Encyclopedia, Inc., New Yo
pp. 579–587.

Halde, C., E. T. Wright, W. H. Pollard, II, V. D. Newcomer, and T. H. S
The Effect of Amphotericin B upon the Yeast Flora of the Gastrointest
of Man, "Antibiotics Annual 1956–1957," Medical Encyclopedia, Inc., N
1957, pp. 123–127.

Louria, D. B., N. Feder, and C. W. Emmons: Amphotericin B in Experimen
plasmosis and Cryptococcosis, "Antibiotics Annual 1956–1957," Medical
pedia, Inc., New York, 1957, pp. 870–877.

Vandeputte, J., J. L. Wachtel, and E. T. Stiller: Amphotericins A and B. A
Antibiotics Produced by a Streptomycete. II. The Isolation and Pro
the Crystalline Amphotericins, "Antibiotics Annual 1955–1956," Medical
pedia, Inc., New York, 1956, pp. 587–591.

### Bacitracin

er, H. S., B. A. Johnson, J. Goldberg, and F. L. Meleney: Bacitracin: Methods of Production, Concentration, and Partial Purification, with a Summary of the Chemical Properties of Crude Bacitracin, *Jour. Bact.*, **55**: 249–255 (1948).

tz, E.: "Polymyxin, Neomycin, Bacitracin," Medical Encyclopedia, Inc., New York, 1956.

son, B. A., H. Anker, and F. L. Meleney: Bacitracin: A New Antibiotic Produced by a Member of the *B. subtilis* Group, *Science*, **102**(2650): 376–377 (1945).

### Chloramphenicol

, Q. R.: Isolation and Characterization of Chloromycetin, *Jour. Biol. Chem.*, **72**: 445–450 (1948).

E. A., and H. P. Todd: A Comparison of Eight Antibiotic Agents, *Jour. Bact.*, **8**: 61–72 (1949).

r, H. E., D. Gottlieb, and H. W. Anderson: Chloromycetin and Streptothricin, *cience*, **107**: 113 (1948).

ch, J., Q. R. Bartz, R. M. Smith, D. A. Joslyn, and P. R. Burkholder: Chloromycetin, a New Antibiotic from a Soil Actinomycete, *Science*, **106**(2757): 417 Oct. 31, 1947).

–, D. Gottlieb, P. R. Burkholder, L. E. Anderson, and T. G. Pridham: *Streptomyces venezuelae*, N. S. P., the Source of Chloromycetin, *Jour. Bact.*, **56**: 467–477 1948).

el, J. E., T. E. Woodward, H. L. Ley, Jr., C. B. Philip, and R. Traub: Chloromycetin in the Treatment of Scrub Typhus, *Science*, **108**(2798): 160–161 (1948).

, G. N., C. S. Worrel, and A. L. Swanson: Inhibition of Bacterial Esterases by hloramphenicol (Chloromycetin), *Jour. Bact.*, **58**: 803 (1949).

, R. M., D. A. Joslyn, O. M. Gruhzit, I. W. McLean, Jr., M. A. Penner, and Ehrlich: Chloromycetin: Biological Studies, *Jour. Bact.*, **55**: 425–448 (1948).

ward, T. E., and C. Wisseman: "Chloramphenicol," Medical Encyclopedia, Inc., ew York, 1957.

### Cycloserine

n, I. G., K. G. S. Nair, and L. J. Boyd: The Treatment of Human Tuberculosis h Cycloserine: A Year's Progress, "Antibiotics Annual 1955–1956," Medical cyclopedia, Inc., New York, 1956, pp. 141–147.

, R. L., P. H. Hidy, and E. K. LaBaw: Cycloserine. I. A Preliminary Report, *tibiotics & Chemotherapy*, **5**: 204 (1955).

, L., R. Strang, L. J. Boyd, M. Schwimmer, and M. G. Mullinos: Cycloserine the Treatment of Nontuberculous Infections, "Antibiotics Annual 1955–1956," dical Encyclopedia, Inc., New York, 1956, pp. 158–164.

ll, M., and A. Prigot: Clinical Trial of Cycloserine in Donovanosis, Lympho-nuloma Venereum and Gonorrhea, "Antibiotics Annual 1955–1956," Medical cyclopedia, Inc., New York, 1956, pp. 153–157.

k, E. H., and P. K. Nenashev: Cycloserine and Isoniazid in Reduced Dosage mbination in the Therapy of Pulmonary Tuberculosis, "Antibiotics Annual 6–1957," Medical Encyclopedia, Inc., New York, 1957, pp. 431–436.

H., L. E. Putnam, and W. A. Randall: Antibacterial Activity and Blood and ne Concentrations of Cycloserine, a New Antibiotic, Following Oral Admin-ation, *Antibiotic Med.*, **1**: 72–79 (1955).

### Erythromycin

n, R. C., P. N. Harris, and K. K. Chen: The Toxicity and Distribution of ycin," *Jour. Am. Pharm. Assoc.* (*Sci. Ed.*), **41**(10): 555–558 (1952).

ous: Erythromycin: Notes on a New Antibiotic, *Scope*, 3(9): 7–9 (1952).

L., and J. M. McGuire: U.S. Patent 2,653,889, Sept. 29, 1953.

Clark, R. K., Jr.: The Chemistry of Erythromycin. I. Acid Degradation *Antibiotics & Chemotherapy,* **3**: 663 (1953).

Herrell, W. E.: "Erythromycin," Medical Encyclopedia, Inc., New York,

Josselyn, L. E., and J. C. Sylvester: Absorption of Erythromycin, *Ant Chemotherapy,* **3**: 63–66 (1953).

McGuire, J. M., R. L. Bunch, R. C. Anderson, H. E. Boaz, E. H. Fly Powell, and J. W. Smith: "Ilotycin," a New Antibiotic, *Antibiotics therapy,* **2**: 281–283 (1952).

Murphy, H. W.: Esters of Erythromycin, "Antibiotics Annual 1953–1954, Encyclopedia, Inc., New York, 1954.

Stephens, V. C.: Esters of Erythromycin. II. Derivatives of Cyclic A "Antibiotics Annual 1953–1954," Medical Encyclopedia, Inc., New York

Sylvester, J. C., and L. E. Josselyn: Absorption of Erythromycin. II. Ery Stearate, *Antibiotics & Chemotherapy,* **3**: 930–933 (1953).

### FUMAGILLIN

Anonymous: New Antibiotic Has Specific Direct Amebicidal Action, *Wh* No. 176, 1953, pp. 2–5.

Eble, T. E., and F. R. Hanson: Fumagillin, an Antibiotic from *Aspergillus f* H-3, *Antibiotics & Chemotherapy,* **1**(1): 54–58 (1951).

Hanson, F. R., and T. E. Eble: An Antiphage Agent Isolated from *Asperg Jour. Bact.,* **58**: 527–529 (1949).

Killough, J. H., G. B. Magill, and R. C. Smith: The Treatment of Amebia Fumagillin, *Science,* **115**: 71 (Jan. 18, 1952).

McCowen, M. C., M. E. Callender, and J. F. Lawlis, Jr.: Fumagillin (H-3): Antibiotic with Amebicidal Properties, *Science,* **113**: 202–203 (1951).

### NEOMYCINS

Jawetz, E.: "Polymyxin, Neomycin, Bacitracin," Medical Encyclopedia, In York, 1956, pp. 36–49.

Waksman, S. A.: "Neomycin," Rutgers University Press, New Brunswick, N.

——— and H. A. Lechevalier: Neomycin, a New Antibiotic Active against mycin-resistant Bacteria, Including Tuberculosis Organisms, *Science,* 10 307 (1949).

### NOVOBIOCIN

Anonymous: Albamycin: A Valuable New Antibiotic, *Scope,* **4**(10): 1–5 (195

Cook, E., G. Eastman, and P. Bunn: The Use of Novobiocin in the Manage Acute Pneumococcal and Staphylococcal Infections, "Antibiotics Annua 1957," Medical Encyclopedia, Inc., New York, 1957, pp. 396–401.

Finland, M.: Discussion of Papers on Cathomycin, "Antibiotics Annual 195 Medical Encyclopedia, Inc., New York, 1956, p. 929.

Frost, B. M., M. E. Valiant, L. McClellan, M. Solotorovsky, and A. C. Cuckle Antimicrobial Activity of Cathomycin, A New Antibiotic, "Antibiotics 1955–1956," Medical Encyclopedia, Inc., New York, 1956, pp. 918–923.

Hinman, J. W., H. Hoeksema, E. L. Caron, and W. G. Jackson: The Partial ture of Novobiocin (Streptonivicin). II. *Jour. Am. Chem. Soc.,* **78**: 1072

Hoeksema, H., M. E. Bergy, W. G. Jackson, J. W. Shell, J. W. Hinman, Fonken, G. A. Boyack, E. L. Caron, J. H. Ford, W. H. DeVries, and Crumi: Streptonivicin, a New Antibiotic. II. Isolation and Characteri *Antibiotics & Chemotherapy,* **6**: 143 (1956*a*).

———, E. L. Caron, and J. W. Hinman: Novobiocin. III. The Structure of biocin, *Jour. Am. Chem. Soc.,* **78**: 2019 (1956*b*).

———, J. L. Johnson, and J. W. Hinman: Structural Studies on Streptoniv New Antibiotic, *Jour. Am. Chem. Soc.,* **77**: 6710 (1955).

Larson, E. J., N. D. Connor, O. F. Swoap, R. A. Runnells, M. C. Prestrud, T. E W. A. Freyburger, W. Veldkamp, and R. M. Taylor: Novobiocin, a New biotic. VI. Toxicology, *Antibiotics & Chemotherapy,* **6**: 226–230 (1956).

r. I. M., and R. B. Walton: A Synthetic Medium for the Production of Novo-
iocin by *Streptomyces spheroides*, "Antibiotics Annual 1956–1957," Medical En-
yclopedia, Inc., New York, 1957, pp. 1040–1045.

ı, C. G., A. Dietz, W. T. Sokolski, and G. M. Savage: Streptonivicin, a New
ıntibiotic. I. Discovery and Biologic Studies, *Antibiotics & Chemotherapy*,
: 135 (1956).

on, H. M., and H. E. Lind: Novobiocin in the Treatment of Urinary Tract In-
ections, "Antibiotics Annual 1956–1957," Medical Encyclopedia, Inc., New York,
57, pp. 420–423.

k, H., D. A. Harris, M. A. Regan, M. Ruger, and H. B. Woodruff: Discovery
d Antimicrobial Properties of Cathomycin, a New Antibiotic Produced by
*eptomyces spheroides* N. Sp., "Antibiotics Annual 1955–1956," Medical Ency-
opedia, Inc., New York, 1956, pp. 909–917.

, H., and W. W. Wright: The Common Identity of Cathomycin and Strepto-
vicin, *Antibiotics & Chemotherapy*, **5**: 670 (1955).

### Nystatin

erg, G. M., W. Weiss, and H. F. Flippin: Microbiological Studies and Pre-
ninary Experiences with Nystatin. I. In Vitro Susceptibility of *Candida albi-
ns*, "Antibiotics Annual 1955–1956," Medical Encyclopedia, Inc., New York,
56, pp. 837-841.

E. L., and R. Brown: Fungicidin, an Antibiotic Produced by a Soil Actino-
cete, *Proc. Soc. Exptl. Biol. Med.*, **76**: 93 (1951).

, N. H., N. Kendall, A. J. Lamberti, and R. H. High: Effects of Nystatin on
l Moniliasis in Infants, "Antibiotics Annual 1955–1956," Medical Encyclo-
lia, Inc., New York, 1956, pp. 711–718.

ans, W. F., C. Bonissol, E. V. Davis, and G. Rake: Nystatin, an Antibiotic
ful for the Control of Fungal and Yeast Contaminants in Tissue Culture,
ntibiotics Annual 1955–1956," Medical Encyclopedia, Inc., New York, 1956,
690–696.

R. A., and H. A. Lechevalier: Survey of the Production of Polyenic Sub-
ces by Soil *Streptomyces*, "Antibiotics Annual 1955-1956," Medical Encyclo-
a, Inc., New York, 1956, pp. 249–254.

n, R. C. V.: *Candida albicans* Infection of the Skin and Mucous Membranes
ted with Nystatin, "Antibiotics Annual 1955–1956," Medical Encyclopedia,
New York, 1956, pp. 851–855.

I.: In Vivo and In Vitro Effect of Nystatin in *Endamoeba histolytica*, "Anti-
cs Annual 1955–1956," Medical Encyclopedia, Inc., New York, 1956, pp. 697–

Tetracycline, Nystatin, and Novobiocin Combinations in Amebiasis, "Anti-
cs Annual 1956–1957," Medical Encyclopedia, Inc., New York, 1957, pp. 175–

. A., and J. F. Pagano: Resistance Studies with Nystatin, "Antibiotics
al 1955–1956," Medical Encyclopedia, Inc., New York, 1956, pp. 704–710.

E. T., J. H. Graham, V. D. Newcomer, and T. H. Sternberg: The Use of
atin in the Treatment of Moniliasis, "Antibiotics Annual 1955–1956," Medi-
ncyclopedia, Inc., New York, 1956, pp. 846–850.

### Penicillin

E. P., E. Chain, C. M. Fletcher, A. D. Gardner, N. G. Heatley, M. A.
ngs, and H. W. Florey: Further Observations on Penicillin, *Lancet*, 2: 177–
89 (1941).

R. F., L. M. Whitmore, Jr., W. E. Brown, W. H. Peterson, B. W. Churchill,
Roegner, T. H. Campbell, M. P. Backus, and J. F. Stauffer: Penicillin Pro-
n by Pigment-free Molds, *Ind. Eng. Chem.*, **45**: 768–773 (1953).

us: "The Story of Penicillin," Merck and Company, Inc., Rahway, N.J.

s: Antibiotics, *Research Today*, 2(2): 19 30 (1945).

s: Biochemistry of Penicillin and Other Antibiotics, *Research Today*,
31–36 (1945).

Anonymous: "Penicillin: Its Properties, Uses, and Preparation," The Pha
Press, London, 1946.

Anonymous: Administration and Dosage of Penicillin, *The Bristol Dig*
Issue), **3**(5): 1–20 (1947).

Arnstein, H. R. V., and P. T. Grant: The Biosynthesis of Penicillin.
corporation of Some Amino Acids into Penicillin, *Biochem. Jour.*,
(1954*a*).

——— and ———: 2. The Incorporation of Cystine into Penicillin, *Bioc*
**57**: 360–368 (1954*b*).

——— and ———: The Metabolism of the Penicillia in Relation to Pen
synthesis, *Bact. Revs.*, **20**(3): 133–147 (1956).

Backus, M. P., J. F. Stauffer, and M. J. Johnson: Penicillin Yields from
Strains, *Jour. Am. Chem. Soc.*, **68**: 152–153 (1946).

Barnes, R. B., R. C. Gore, E. F. Williams, S. G. Linsley, and E. Peterson
Analysis of Penicillins, paper presented at Conference on Antibiotic
held at Washington, D.C., on Jan. 31 and Feb. 1, 1947, under the ausp
Antibiotics Study Section, National Institute of Health.

Benedict, R. G., and A. F. Langlykke: Antibiotics, *Ann. Rev. Microbiol.*,
(1947).

Berger, F. M.: Extraction and Purification of Penicillin, *Nature*, **154**: 459

Bowden, J. P., and W. H. Peterson: The Role of Corn Steep Liquor in
of Penicillin, *Arch. Biochem.*, **9**(3): 387–399 (1946).

Brunel, J.: Qui a découvert la pénicilline? *Rev. can. biol.*, **3**: 333-343 (1944

Callahan, J. R.: Penicillin Large-scale Production by Deep Fermentatio
*Eng.*, **51**(4): 94–98 (1944).

———: Penicillin by Deep Fermentation, *Chem. Eng.*, **51**(4): 130–133 (19

Catch, J. R., A. H. Cook, and I. M. Heilbron: Purification and Chemistry
cillin, *Nature*, **150**: 633–634 (1942).

Chain, E., and H. W. Florey: Penicillin, *Endeavour*, Vol. 3, No. 9, 1944.

———, ———, A. D. Gardner, N. G. Heatley, M. A. Jennings, J. Orr-E
A. G. Sanders: Penicillin as a Chemotherapeutic Agent, *Lancet*, **2**:
(1940).

Challinor, S. W.: Production of Penicillin, *Nature*, **150**: 688 (1942).

——— and J. MacNaughton: The Production of Penicillin, *Jour. Path.*
441–446 (1943).

Clutterbuck, P. W., R. Lovell, and H. Raistrick: Studies in the Biochem
Micro-organisms. XXVI. The Formation from Glucose by Membe
*Penicillium chrysogenum* Series of a Pigment, an Alkali-soluble Pro
Penicillin—The Antibacterial Substance of Fleming, *Biochem. Jour.*,
1918 (1932).

Coghill, R. D.: Penicillin Science's Cinderella, *Chem. Eng. News*, 22(8):
(1944).

———, A. E. Osterberg, and G. R. Hazel: The Relative Effectiveness of P
cillins G, X, and K, *Science*, **103**: 709–710 (1946).

Committee on Medical Research, O.S.R.D., and the Medical Research
London: Chemistry of Penicillin, *Science*, **102**(2660): 627–629 (1945).

Cook, R. P., W. J. Tulloch, M. B. Brown, and J. Brodie: The Production
cillin on Media Made from Vegetable Extracts, Particularly Extracts
*Jour. Path. Bact.*, **54**: 555 (1944).

Da Rocha, R. V.: Contribuicao ao Estudo da Peniciline, Tese Fac. de Med.
Alegere, Rio Grande do Sul, Brazil, 1943.

Davey, V. G., and M. J. Johnson: Penicillin Production in Corn Stee
with Continuous Carbohydrate Addition, *Appl. Microbiol.*, **50**(4):
(1953).

Dolkart, R. E., F. L. Dey̆, and G. X. Schwemlein: Penicillin Assay Techn
Comparative Study, *Jour. Bact.*, **53**: 17–24 (1947).

Donovick, R., D. Lapedes, and F. Pansy: Studies on the Quantitative D
Analysis of Mixtures of Several Essentially Pure Penicillin Types, *Jo*
**54**(4): 425–442 (1947).

noy, J., and R. Pratt: Cytochemical Mechanisms of Penicillin Action. I. xidation-Reduction Levels, *Jour. Bact.*, **53**: 657–666 (1947).

neaud, V., F. H. Carpenter, R. W. Holley, A. H. Livermore, and J. R. chelle: Synthetic Penicillin, *Science*, **104**(2706): 431–433, 450 (1946).

H.: The Relative Activity of Penicillins F, G, K, and X against Spirochetes d Streptococci In Vitro, *Jour. Bact.*, **52**: 81–88 (1946).

and A. Musselman: The Low Therapeutic Activity of Penicillin K Relative that of Penicillins F, G, and X, and Its Pharmacological Basis, *Science*, **103**: -620 (1946).

al Board of the Monograph on the Chemistry of Penicillin: The Chemical dy of Penicillin: A Brief History, *Science*, **105**(2739): 653–659 (1947).

H., and B. Porzecanski: Inactivation of Penicillin by Zinc Salts, *Science*, (2681): 629–630 (1946).

A. L.: The Commercial Production of Penicillin, *Chem. Ind.*, **54**: 501–504 44).

Penicillin, *Sci. Monthly*, **58**: 405–409 (1944).

W. H.: The Industrial Development of Antibiotics, *Chem. Eng. News*, 18): 1284–1287 (1948).

. A.: On the Antibacterial Action of Cultures of a Penicillium, with Special erence to Their Use in the Isolation of *B. Influenzae*, *Brit. Jour. Pathol.*, **10**: -236 (1929).

On the Specific Antibacterial Properties of Penicillin and Potassium Tellurite, *r. Path. Bact.*, **35**: 831–842 (1932).

Mode of Action of Penicillin, *Nature*, **148**: 757 (1941).

In Vitro Tests of Penicillin Potency, *Lancet*, **1**: 732–733 (1942).

The Discovery of Penicillin, *Brit. Med. Bull.*, 2(1): 4–5 (1944).

"Penicillin. Its Practical Application," McGraw-Hill Book Company, Inc., iston Division, New York, 1946.

H. E., and H. W. Florey: General and Local Administration of Penicillin, *cet*, **1**: 387–397 (1943).

H. W.: Penicillin: Its Development for Medical Uses, *Nature*, **153**: 40–42 4).

. Chain, N. G. Heatley, M. A. Jennings, A. G. Sanders, E. P. Abraham, and . Florey: "Antibiotics," Oxford University Press, New York, 1949.

d M. A. Jennings: Some Biological Properties of Highly Purified Penicillin, *Jour. Exptl. Pathol.*, **23**: 120–123 (1942).

W.: Quantitative Estimation of Penicillin, *Jour. Biol. Chem.*, **144**: 285–286 ).

icrobiological Aspects of Penicillin. I. Methods of Assay, *Jour. Bact.*, **46**: 02 (1943).

icrobiological Aspects of Penicillin. IV. Procedure for the Cup Assay of illin, *Jour. Bact.*, **47**: 43–58 (1943).

d E. O. Karow: Microbiological Aspects of Penicillin. VIII. Penicillin from ent Fungi, *Jour. Bact.*, **49**(1): 19–29 (1945).

. B. Woodruff, and L. E. McDaniel: Microbiological Aspects of Penicillin. Production of Penicillin in Surface Cultures of *Penicillium notatum*, *Jour.* **46**: 421–433 (1943).

——, and ———: Microbiological Aspects of Penicillin. IV. Production nicillin in Submerged Cultures of *Penicillium notatum*, *Jour. Bact.*, **51**: 465– 946).

——, D. Perlman, L. E. McDaniel, B. L. Wilker, and D. Hendlin: Micro- ical Aspects of Penicillin. IX. Cottonseed Meal as a Substitute for Corn Liquor in Penicillin Production, *Jour. Bact.*, **51**: 695–698 (1946).

B., J. J. Stefaniak, B. H. Olson, and M. J. Johnson: A Comparison of lin-producing Strains of *Penicillium notatum-chrysogenum*, *Jour. Bact.*, 9-140 (1946).

. R., and A. A. Levi: A Microchromatographic Method for the Detection pproximate Determination of the Different Penicillins in a Mixture, , **158**: 675–678 (1946).

——— and ———: A Micro-chromatographic Method for the Detection
mate Determination of the Different Penicillins in a Mixture, *Ana*
288 (1947).

Hansen, H. N.: Observations on the Mechanism of Action of Penicillin
281–285 (1942).

——— and W. C. Snyder: Relation and Dual Phenomenon in *Penicill*
to Penicillin Production, *Science*, **99**: 264–265 (1944).

Herrell, W. E.: "Penicillin and Other Antibiotic Agents," W. B. Saunde
Philadelphia, 1946.

Higuchi, K., F. G. Jarvis, W. H. Peterson, and M. A. Johnson: Effect of
Acid Derivatives on the Types of Penicillin Produced by *Penicilliu*
*num* Q176, *Jour. Am. Chem. Soc.*, **68**: 1669–1670 (1946).

——— and W. H. Peterson: Estimation of Types of Penicillin in Brot
ished Products. A Microbiological Method, *Anal. Chem.*, **19**(1): 68-

Hobby, G. L., T. F. Lenert, and B. Hyman: The Effect of Impurities on
therapeutic Action of Crystalline Penicillin, *Jour. Bact.*, **54**(3): 305-

Hosler, P., and M. J. Johnson: Penicillin from Chemically Defined Medi
*Chem.*, **45**: 871 (1953).

Hunter, A. C., and W. A. Randall: Standardization of Assay of Peni
*Assoc. Offic. Agr. Chemists*, **27**(3): 430–438 (1944).

Johnson, M. J.: Metabolism of Penicillin-Producing Molds, *Ann. N.Y. Ac*
(Art. 2): 57–66 (1946).

Joslyn, D. A., Penicillin Assay: An Outline of Four Hour Turbidimetr
*Science*, **99**: 21: 1944.

Kavanagh, F.: A Qualitative Test for Penicillin, *Science*, **105**: 189 (1947).

———: Antibacterial Substances from Fungi and Green Plants, *Advance*
*mol.*, **7**: 461–511 (1947).

Keefer, C. S., et al., Committee on Therapeutics, National Research Counc
lin in the Treatment of Infections, *Jour. Am. Med. Assoc.*, **122**: 1217 (

Knight, S. G., and W. C. Frazier: The Control of Contaminants in Peni
mentations by Antiseptic Chemicals, *Jour. Bact.*, **50**: 505–516: 1945.

Kocholaty, W., Cultural Characteristics of *Penicillium notatum* in Relati
Production of Antibacterial Substances: Indication of the Dual Natu
Antibacterial Substance, *Jour. Bact.*, **44**: 469–477: 1942.

Koffler, H. E., R. L. Emerson, D. Perlman, and R. H. Burris: Chemical C
Submerged Penicillin Fermentations, *Jour. Bact.*, **50**: 517–548 (1945).

———, S. G. Knight, and W. C. Frazier: The Effect of Certain Mineral
on the Production of Penicillin in Shake Flasks, *Jour. Bact.*, **52**(1)
(1947).

———, ———, R. L. Emerson, and R. H. Burris: The Effect of Certain Che
Penicillin Production and Mold Metabolism in Shake Flask Fermentati
*Bact.*, **50**: 549–559 (1945).

Kolmer, J. A.: "Penicillin Therapy, Including Streptomycin, Tyrothricin
Antibiotic Therapy," 2d ed., Appleton-Century-Crofts, Inc., New York

Lederle: "Excerpts and Conclusions from the Literature on the Principal
Penicillin," Lederle Laboratories Division, American Cyanamid Co., N
1948.

Lee, S. W., E. J. Foley, J. A. Epstein, J. H. Wallace, Jr.: Improvemen
Turbidimetric Assay for Penicillin, *Jour. Biol. Chem.*, **152**: 485–486 (19

Libby, R. L., and N. L. Holmberg: The Activity of Penicillins G and X
*Science*, **102**: 303–304 (1945).

McMahan, J. R.: An Improved Short Time Turbidimetric Assay for Penici
*Biol. Chem.*, **153**: 249–258 (1944).

Moore, J. E.: "Penicillin in Syphilis," Charles C Thomas, Publisher, Spring
1946.

Moyer, A. J., and R. D. Coghill: Penicillin. VIII. Production of Penicillin i
Cultures, *Jour. Bact.*, **51**(1): 57–78 (1946*a*).

——— and ———, Penicillin IX. The Laboratory Scale Production of Pen

ubmerged Culture by *Penicillium notatum* Westling (NRRL 832), *Jour. Bact.*,
1(1): 79–93 (1946*b*).

- and ———: Penicillin X. The Effect of Phenylacetic Acid on Penicillin Pro-
uction, *Jour. Bact.*, **53**(3): 329–341 (1947).

l. R. G.: "Antibiotic Substances, Their Biological and Chemical Properties,"
l ed., National Institute of Health, Division of Research Grants and Fellow-
ips, Antibiotics Study Section, Bethesda, Md., February, 1948.

nal Academy of Sciences: "The Chemistry of Penicillin," Princeton University
ress, Princeton, N.J., 1948.

. M., M. Meads, and M. Finland: Penicillin X, *Jour. Am. Med. Assoc.*, **129**:
7-261 (1945).

I. A., and J. W. Appling: Penicillin Production, *Science* (*N.S.*), **100**: 51 (1944).

R.: Influence of Phosphate on Stability of Crude Penicillin, *Plant Physiol.*,
(3): 308–314 (1947*a*).

: Influence of Phosphate on Stability of Partially Purified Penicillins, *Jour.*
*. Pharm. Assoc., Sci. Ed.*, **36**(3): 69–72 (1947*b*).

and J. Dufrenoy: Practical Three-hour and Two-hour Cylinder-Plate Assays
Penicillin, *Nature*, **159**: 576 (1947).

and ———: Cytochemical Interpretation of the Mechanism of Penicillin
tion, *Bact. Revs.*, **12**(1): 79–103 (1948).

and ———: "Antibiotics," 2d ed., J. B. Lippincott Company, Philadelphia,
3.

———, and L. A. Strait: The Enhancement of Penicillin Effectiveness in
o by Traces of Cobalt, *Jour. Bact.*, **55**(1): 75–77 (1948).

t. S. C., and C. G. Dunn: "Industrial Microbiology," 2d ed., McGraw-Hill
k Company, Inc., New York, 1949.

., W. B. Dunham, and R. Donovick: The Evaluation of Antibiotics by the
vention of Experimental Syphilis, *Jour. Infectious Diseases*, **81**: 122–129
47).

and A. P. Richardson: Pharmacology of Penicillin, *Ann. N.Y. Acad. Sci.*, **48**
. 2): 143–174 (1946).

kamp, C. H.: A Method for Determining the Concentration of Penicillin in
y Fluids and Exudates, *Proc. Soc. Exptl. Biol. Med.*, **51**: 95–97 (1942).

. B.: The Development of Improved Penicillin-producing Molds, *Ann. N.Y.*
*l. Sci.*, **48** (Art. 2): 41–56 (1946).

A Decade of Antibiotics in America, *Mycologia*, **44**(1): 1–59 (1952).

nd D. F. Alexander: The Preservation of Molds by the Lyophil Process,
*ologia*, Vol. 37, July–August, 1945.

nd ———: Penicillin. V. Mycological Aspects of Penicillin Production.
*Elisha Mitchell Sci. Soc.*, **61**: 74–113 (1945).

———, and R. D. Coghill: Penicillin. II. Natural Variation and Penicillin
uction in *Penicillium notatum* and Allied Species, *Jour. Bact.*, **48**(6): 639–
1944).

d R. D. Coghill: "Home-made" Penicillin, *Jour. Am. Med. Assoc.*, **123**: 1135
5).

nd D. I. Fennell: The Production of Penicillin X in Submerged Culture,
*. Bact.*, **51**: 761–777 (1946).

M. D., and W. H. Schmidt: Penicillin. IV. A Device for Placing Cylinders
say Plates, *Jour. Bact.*, **49**(4): 395–400 (1945).

A Study of the Bactericidal, Bacteriolytic or Inhibitory Substance Produced
me Molds and Some Factors Which Influence Its Production, *Jour. Bact.*,
1 (1933).

he Ability of a Mold or Its Metabolic Products to Inhibit Bacterial
th, *Jour. Bact.*, **27**: 28 (1934).

me Properties of a Bacterial Substance Produced by a Mold, *Jour. Bact.*,
5–221 (1935).

. C., C. K. Cain, R. D. Muir, F. J. Reithel, W. L. Goby, J. T. Van Brug-
. M. Homan, P. A. Katzman, L. R. Jones, and E. A. Doisy: Penicillin B,

An Antibacterial Substance from *Penicillium notatum, Jour. Biol.* 47–58 (1943).

Salivar, C. J., V. V. Bogert, and E. V. Brown: Separation and Identifica icillin Species in Commercial Penicillin (Q-176 Culture), paper Conference on Antibiotic Research, Washington, Jan. 31–Feb. 1, 1947 pices of the National Institute of Health, Antibiotics Study Section.

Savage, G. M., and M. J. Vander Brook: The Fragmentation of the *Penicillium notatum* and *Penicillium chrysogenum* by a High Speed the Evaluation of Blended Seed, *Jour. Bact.,* **52**: 385–391 (1946).

Schmidt, W. H., and A. J. Moyer: Penicillin. I. Methods of Assay, **47**(2): 199–210 (1944).

———, G. E. Ward, and R. D. Coghill: Penicillin. VI. Effect of Dissocia of *Bacillus subtilis* on Penicillin Assay, *Jour. Bact.,* **49**: 411–412 (1945

Sheehan, J. C., W. J. Mader, and D. J. Cram: A Chemical Assay Method lin G, *Jour. Am. Chem. Soc.,* **68**: 2407 (1946).

Smith, E. C.: *Jour. Soc. Chem. Ind.,* **65**: 308–314 (1946).

Smith, L.: The Bacteriostatic Agent of *Penicillium chrysogenum, Jour. Fra* **234**: 396–402 (1942).

Sollmann, T.: "A Manual of Pharmacology and Its Applications to Therap Toxicology," 7th ed., W. B. Saunders Company, Philadelphia, 1948.

Soltero, F. V., and M. J. Johnson: The Effect of the Carbohydrate Nu Penicillin Production by *Penicillium chrysogenum* Q-176, *Appl. Microb* 52–57 (1953).

Srinivasa, R. S.: Production of Penicillin, *Nature,* **154**: 83 (1944).

Stefaniak, J. J., F. B. Gailey, C. S. Brown, and M. J. Johnson: Pilot Pla ment for Submerged Production of Penicillin, *Ind. Eng. Chem.,* 38 (1946).

———, ———, F. G. Jarvis, and M. J. Johnson: The Effect of Environme ditions on Penicillin Fermentations with *Penicillium chrysogenum* X-1 *Bact.,* **52**: 119–127 (1946).

Stone, R .W., and M. A. Farrell: Synthetic Media for Penicillin Production **104**(2706): 445–446 (1946).

Strait, L. A., J. Dufrenoy, R. Pratt, and V. Lamb: Enhancement of Penicil tiveness by Traces of Cobalt, *Jour. Am. Pharm. Assoc., Sci. Ed.,* 37 (1948).

Taylor, H. G.: Growth of *Penicillium notatum* on Various Media and the ment of An Antibacterial Substance, *Proc. Soc. Exptl. Biol. Med.,* 52 (1943).

Taylor, T. H. M.: Penicillin, *Chem. Eng. Progr., Trans. Sec.,* **43**(4): (1947).

Thom, C., and K. B. Raper: "A Manual of the Aspergilli," The Williams & Company, Baltimore, 1945.

Tompsett, R., S. Shultz, and W. McDermott: The Relations of Protein Bi the Pharmacological and Antibacterial Activity of Penicillins X, G, Di and K, *Jour. Bact.,* **53**: 581–595 (1947).

Van Winkle, W., and R. P. Herwick: Penicillin—A Review, *Jour. Am. Pharm* *Sci. Ed.,* **34**: 97–109 (1945).

Vervey, W. F., and A. K. Miller: The Effect of Caronamide upon Penicillin of Experimental Pneumococcus and Typhoid Infections in Mice, paper p at Conference on Antibiotic Research, Washington, Jan. 31–Feb. 1, 194 the auspices of National Institute of Health, Antibiotics Study Section.

——— and ———: The Effect of Caronamide upon Penicillin Therapy of mental Pneumococcus and Typhoid Infections in Mice, *Jour. Bact.,* (1947).

Waksman, S. A.: "Microbial Antagonisms and Antibiotic Substances," 2d e Commonwealth Fund, New York, 1947.

——— and E. Horning: Distribution of Antagonistic Fungi in Nature an Antibiotic Activity, *Mycologia,* **35**: 47–65 (1943).

——— and H. C. Reilly: Strain Specificity and Production of Antibiotic Sub

II. *Penicillium notatum-chrysogenum* group. *Proc. Nat. Acad. Sci.*, **30**: 99–105
1944).

h. H., R. H. Herwick, and A. C. Hunter: Penicillin: Properties and Standardiza-
ion *Jour. Am. Pharm. Assoc., Pract. Pharm. Ed.*, **6**(4): 93–96 (1945).

–, L. E. Putnam, W. A. Randall, and R. P. Herwick: Penicillin X: Successful
reatment of Gonorrhea in a Single Intramuscular Injection, *Jour. Am. Med.
ssoc.*, **126**: 1024 (1944).

–, W. A. Randall, and L. Knudsen: Methods of Testing Antibiotic Substances
nd Limitations Involved, *Jour. Am. Pharm. Assoc., Sci. Ed.*, **35**(4): 102–113
1946).

er, H. A.: Penicillin and Streptomycin—A Review of Their Current Uses, *Jour.
ansas Med. Soc.*, June, 1947.

en, A. J., and G. M. Savage: The Relation of Natural Variation in *Penicillium
otatum* to the Yield of Penicillin in Surface Culture, *Jour. Bact.*, **53**: 231–240
947).

, A. G. C., L. O. Krampitz, and C. H. Werkman: On a Synthetic Medium for
e Production of Penicillin, *Arch. Biochem.*, **8**: 303–311 (1945).

ruff, H. B., and J. W. Foster: Microbiological Aspects of Penicillin. VII. Bac-
rial Penicillinase, *Jour. Bact.*, **49**: 7–17 (1944).

## POLYMYXINS

orth, G. C., A. M. Brown, and G. Brownlee: Aerosporin, an Antibiotic Pro-
iced by *Bacillus aerosporus* Greer, *Nature*, **160**: 263 (1947).

ict, R. G., and A. F. Langlykke: Antibiotic Activity of *Bacillus polymyxa*, *Jour.
ct.*, **54**: 24–25 (1947).

G. J.: The Influence of Antibiotics on Some Biological Contaminants in the
ewery, *Am. Soc. Brewing Chemists, Proc.*, 1955, pp. 43–55.

. E.: "Polymyxin, Neomycin, Bacitracin," Medical Encyclopedia, Inc., New
rk, 1956, pp. 11–35.

P. H.: Antibiotics Derived from *Bacillus polymyxa*, *Ann. N.Y. Acad. Sci.*,
(Art. 5): 855–1000 (1949).

., and J. Dufrenoy: "Antibiotics," 2d ed., J. B. Lippincott Company, Phila-
phia, 1953, pp. 190–194.

. P. G., R. G. Shepherd, and H. G. White: Polymyxin: A New Chemothera-
tic Agent, *Bull. Johns Hopkins Hosp.*, **81**: 43–54 (1947).

ov, F. B., and J. B. Bockelmann: Antibiotics as Inhibitors of Microbiological
tamination in Beer, *Jour. Agr. Food Chem.*, **1**(20): 1219–1222 (1953).

## RISTOCETIN

, W. E., E. L. Alford, E. J. Rdzok, and J. C. Sylvester: Ristocetin: The Devel-
ent of Resistance and Bactericidal Activity, "Antibiotics Annual 1956–1957,"
dical Encyclopedia, Inc., New York, 1957*a*, pp. 693–698.

A. C. Sinclair, R. J. Theriault, A. W. Goldstein, C. J. Rickher, H. B. Warren,
T. J. Oliver, and J. C. Sylvester: Ristocetin: Microbiologic Properties, "Anti-
ics Annual 1956–1957," Medical Encyclopedia, Inc., New York, 1957*b*, pp. 687–

J. E., J. R. Schenck, and M. P. Hargie: Ristocetins A and B: Two New Anti-
ics, Isolation and Properties, "Antibiotics Annual 1956–1957," Medical Ency-
edia, Inc., New York, 1957, pp. 699–705.

ky, M. J., B. M. Limson, and J. E. Hawkins: Ristocetin: A New Antibiotic—
oratory and Clinical Studies, a Preliminary Report, "Antibiotics Annual
–1957," Medical Encyclopedia, Inc., New York, 1957, pp. 706–715.

## STREPTOMYCIN

er, H. E., G. Leidy, G. Rake, and R. Donovick: Hemophilus Influenzae Men-
is Treated with Streptomycin, *Jour. Am. Med. Assoc.*, **132**: 434–440 (1946).

, R. G., and A. F. Langlykke: Antibiotics, *Ann. Rev. Microbiol.*, **1**: 193–236
7).

Bennett, R. E.: Nutrition of *Streptomyces griseus* in Relation to Streptomy *Jour. Bact.*, **53**: 254 (1947).

Bondi, A., Jr., C. C. Dietz, and E. H. Spaulding: Interference with the Anti Action of Streptomycin by Reducing Agents. *Science*, **103**: 399–401 (194

Boxer, G. E., V. C. Jelinek, and P. M. Leghorn: The Colorimetric Deter of Streptomycin in Clinical Preparations, Urine and Broth, *Jour. Biol* **169**: 153–165 (1947).

Brink, N. G., F. A. Kuehl, Jr., and K. Folkers: Streptomyces Antibio Degradation of Streptomycin to Streptobiosamine Derivatives, *Science*, 1 507 (1945).

———, ———, E. H. Flynn, and K. Folkers: Streptomyces Antibiotics. Structure of Tetraacetylbisdesoxystreptobiosamine, *Jour. Am. Chem. S* 2405 (1946).

———. ———, ———, and ———: Streptomyces Antibiotics. XII. The Deg of Streptomycin and Dihydrostreptomycin with Methanol, *Jour. Am. Che* **68**: 2557 (1946).

Carter, H. E., R. K. Clark, Jr., S. R. Dickman, Y. H. Loo, P. S. Skell, and Strong: Isolation and Purification of Streptomycin, *Jour. Biol. Chem.*, 16 342 (1945).

Carvajal, F.: Studies on the Structure of *Streptomyces griseus, Mycologia*, 3 595 (1946).

Committee on Chemotherapeutics and Other Agents, National Research C C. S. Keefer, Chairman: Streptomycin in the Treatment of Infections, *Jou Med. Assoc.*, **132**: 4–11, 70–77 (1946).

Denkelwater, R., M. A. Cook, and M. Tishler: The Effect of Cysteine on St mycin and Streptothricin, *Science*, **102**: 12 (1945).

Donovick, R., D. Hamre, F. Kavanagh, and G. Rake: A Broth Dilution Meth Assaying Streptothricin and Streptomycin. *Jour. Bact.*, **50**: 623-628 (1945).

——— and G. Rake: Influence of Certain Substances on Activity of Strepto I. Modifications in Test Medium, *Proc. Soc. Exptl. Biol. Med.*, **61**: 2 (1946).

——— and ———: Studies on Some Biological Aspects of Dihydrostrepto *Jour. Bact.*, **53**(2): 205–211 (1947).

———, ———, and J. Fried: The Influence of Certain Substances on the Anti Activity of Streptomycin In Vitro. II. The Action of Some Carbonyl Rea on Streptomycin, *Jour. Biol. Chem.*, **164**(1): 173–181 (1946).

Engels, W. H.: The Industrial Production of Streptomycin, paper present A.A.A.S. Symposium on Antibiotics, Chicago, Dec. 29–30, 1947.

———: The Industrial Development of Antibiotics, *Chem. Eng. News*, **26**(18): 1287 (1948).

Fried, J., G. A. Boyack, and O. Wintersteiner: Streptomycin: The Chemical N of Streptidine, *Jour. Biol. Chem.*, **162**(2): 391–392 (1946).

——— and H. E. Stavely: Streptomycin. V. Degradation of Streptomycin Streptidine, Streptobiosamine and D-Mannose, *Jour. Am. Chem. Soc.*, **69**: (1947).

——— and E. Titus: Streptomycin B, An Antibiotically Active Constituent of S tomycin Concentrates, *Jour. Biol. Chem.*, **168**(1): 391–392 (1947).

———, D. E. Walz, and O. Wintersteiner: Streptomycin. III. 4-Desoxy-L-Eryt (Threose) Phenylosazone from Streptobiosamine, *Jour. Am. Chem. Soc.*, **68**: (1946).

——— and O. Wintersteiner: The Composition of Streptomycin Reineckate. *Sc* **104**: 273–274 (1946).

——— and ———: Streptomycin. II. Reduction and Oxidation Products of Str mycin and of Streptobiosamine, *Jour. Am. Chem. Soc.*, **69**: 79–86 (1947).

Geiger, W. B., S. R. Green, and S. A. Waksman: The Inactivation of Strepto and Its Practical Applications, *Proc. Soc. Exptl. Biol. Med.*, **61**: 187–192 (1

Gordon, A. M.: Streptomycin in Tularemia, *Jour. Am. Med. Assoc.*, 132: (1946).

heb, D., and H. W. Anderson: Morphological and Physiological Factors in
treptomycin Production, *Bull. Torrey Botan. Club*, **74**(4): 293–302 (1947).

gy, P., and H. F. Lee: Chemotherapy: Penicillin, Sulfonamides, Streptomycin,
nd Tyrothricin, *Advances in Pediat.*, **2**: 151–193 (1947).

e, D., G. Rake, and R. Donovick: Bactericidal Action of Streptomycin, *Proc.
oc. Exptl. Biol. Med.*, **62**: 25–31 (1946).

ıan, D. H.: A Method for Estimating the Concentrations of Streptomycin in
ody Fluids, *Proc. Staff Meetings Mayo Clinic*, **20**: 145–150 (May 16, 1945).

ıan, F. R.: Streptomycin in the Treatment of Experimental Infections with
licro-organisms of the Friedlander Group (Klebsiella), *Proc. Staff Meetings
ayo Clinic*, **20**: 32–39 (Feb. 7, 1945).

ll, Wallace E.: "Penicillin and Other Antibiotic Agents," W. B. Saunders Com-
any, Philadelphia and London, 1946. Special reference is made to Chaps. XXIII
ıd XXVI.

eld, J. W., and C. W. Buggs: The Present Status of Streptomycin, *N.Y. State
ur. Med.*, **47**(11): 1276–1283 (1947).

, G. L., and T. F. Lenert: Observations on the Action of Streptomycin *in vitro*
I), *Proc. Soc. Exptl. Biol. Med.*, **65**: 242–249 (1947).

D., H. J. Metzger, A. Schatz, and S. A. Waksman: Control of Gram-Negative
cteria in Experimental Animals by Streptomycin, *Science*, **100**: 103–105
44).

ıtrick, S. D.: A Case Study in Biochemical Engineering, *Chem. Eng.*, **54**(11):
-96 (1947).

: The Story of Streptomycin, *Chem. Eng.*, **54**(11): 97–102 (1947).

F. A., Jr., E. H. Flynn, N. G. Brink, and K. Folkers: Streptomyces Anti-
otics. X. The Degradation of Streptomycin and Dihydrostreptomycin with
hyl Mercaptan, *Jour. Am. Chem. Soc.*, **68**: 2096–2101 (1946).

———, ———, and ———: Streptomyces Antibiotics. XIII. The Structure of
eptobiosamine, *Jour. Am. Chem. Soc.*, **68**: 2679–2684 (1946).

———, F. W. Holly, R. Mozingo, and K. Folkers: Streptomyces Antibiotics.
N-Methyl-*l*-Glucosamine from Streptomycin, *Jour. Am. Chem. Soc.*, **68**: 536
46).

R. L. Peck, C. E. Hoffhine, Jr., R. P. Graber, and K. Folkers: Streptomyces
ibiotics. VII. Isolation of Streptomycin, *Jour. Am. Chem. Soc.*, **68**: 1460–1462
6).

———, ———, E. W. Peel, and K. Folkers: Streptomyces Antibiotics. XIV.
Position of the Linkage of Streptobiosamine to Streptidine in Streptomycin,
. *Am. Chem. Soc.*, **69**: 1234 (1947).

———, A. Walti, and K. Folkers: Streptomyces Antibiotics. I. Crystalline
of Streptomycin and Streptothricin, *Science*, **102**: 34–35 (1945).

D., R. Donovick, and G. Rake: Further Studies on the Testing of Sterility
oncentrated Streptomycin Solutions, *Proc. Soc. Exptl. Biol. Med.*, **64**: 269–271
7).

T. F., and G. L. Hobby: Observations on the Action of Streptomycin *in vitro*
*Proc. Soc. Exptl. Biol. Med.*, **65**: 235–242 (1947).

G. A., and E. Campbell: Preparation of Streptomycin, *Jour. Biol. Chem.*,
163–171 (1945).

. B., P. Schwed, and J. W. Sackett: Polarographic Analysis of Streptomycin,
. *Am. Chem. Soc.*, **68**: 528 (1946).

H., P. S. Skell, H. H. Thornberry, J. Ehrlich, J. M. McGuire, G. M. Savage,
J. C. Sylvester: *Jour. Bact.*, **50**(6): 701–709 (1945).

ıd Company, Inc.: "The Story of Streptomycin," 1946; "The Clinical Use of
tomycin," 1947; "The Effects of Streptomycin in Tuberculosis in Man,"
Rahway, N.J.

H.: Pharmacology of Streptothricin and Streptomycin, *Ann. N.Y. Acad.*
**48** (Art. 2): 101–117 (1946).

R., T. F. Paine, and M. Finland: Streptomycin. I. Bacteriologic and Phar-
logic Effects, *New Eng. Jour. Med.*, **236**: 701–712 (May 8, 1947).

Osgood, E. E., and S. M. Graham: Simple Rapid Method for Assay of Ba and Bacteriostatic Agents, *Am. Jour. Clin. Pathol.,* **17**: 93–107 (1947).

Oswald, E. J., and J. K. Nielsen: Studies on Stability of Streptomycin in *Science,* **105**: 184 (1947).

Paine, T. F., R. Murray, and M. Finland: Streptomycin. II. Clinical U *Eng. Jour. Med.,* **236**: 748–760 (May 15, 1947).

Parke, Davis & Company: "Streptomycin," Detroit, Mich., 1947.

Peck, R. L., N. G. Brink, F. A. Kuehl, Jr., E. H. Flynn, A. Walti, and K. Streptomyces Antibiotics. II. Crystalline Streptomycin Trihydrochlori cium Chloride Double Salt, *Jour. Am. Chem. Soc.,* **67**: 1866 (1945).

———, R. P. Graber, A. Walti, E. W. Peel, C. E. Hoffhine, Jr., and K. Folkers tomyces Antibiotics. IV. Hydrolytic Cleavage of Streptomycin to Str *Jour. Am. Chem. Soc.,* **68**: 29–31 (1946).

———, C. E. Hoffhine, Jr., and K. Folkers: Streptomyces Antibiotics. IX. streptomycin, *Jour. Am. Chem. Soc.,* **68**: 1390 (1946).

———, ———, E. W. Peel, R. E. Graber, F. W. Holly, R. Mozingo, and K. Streptomyces Antibiotics. VII. The Structure of Streptidine, *Jour. Am. Soc.,* **68**: 776–781 (1946).

Porter, R. W.: Streptomycin Engineered into Commercial Production, *Chem* **53**(10): 94–98 (1946).

———: Streptomycin Calcium Chloride Complex, *Merck Rept.,* July, 1947.

Postovskiĭ, I. Ya., and N. P. Bednyagina: Antibiotics, *Uspekhi Khim.,* 1 (1947).

Price, C. W., J. K. Nielsen, and H. Welch: The Estimation of Streptom Body Fluids, *Science,* **103**: 56–57 (1946).

Priest, W. S.: The Present Status of Penicillin and the Newer Antibiotics, *Ind* **15**: 434–436 (1946).

Rake, G., and R. Donovick: A Procedure for Testing Sterility of Conce Streptomycin Solutions, *Proc. Soc. Exptl. Biol. Med.,* **62**: 31–33 (1946).

——— and ———: Studies on the Nutritional Requirements of *Streptomyces* for the Formation of Streptomycin, *Jour. Bact.,* **52**: 223–226 (1946).

——— and ———: Features of the Antibacterial Action of Streptomycin, pap sented at the Meeting of the A.A.A.S., Chicago, Dec. 29, 1947.

———, D. Hamre, F. Kavanagh, W. L. Koerber, and R. Donovick: On the T of Streptomycin, *Am. Jour. Med. Sci.,* **210**: 61–66 (1945).

———, C. M. McKee, F. E. Pansy, and R. Donovick: On Some Biological C teristics of Streptomycin B, *Proc. Soc. Exptl. Biol. Med.,* **65**: 107–112 (19

Regna, P. P., L. A. Wasselle, and I. A. Solomons: Stability of Streptomycin *Biol. Chem.,* **165**: 631–638 (1946).

Robinson, H. J., D. G. Smith, and O. E. Graessle: Chemotherapeutic Proper Streptomycin, *Proc. Soc. Exptl. Biol. Med.,* **57**: 226–231 (1944).

Schatz, A., E. Bugie, and S. A. Waksman: Streptomycin, a Substance Exh Antibiotic Activity Against Gram-positive and Gram-negative Bacteria, *Soc. Exptl. Biol. Med.,* **55**: 66–69 (1944).

——— and S. A. Waksman: Effect of Streptomycin and Other Antibiotic Subs upon *Mycobacterium tuberculosis* and Related Organisms, *Proc. Soc. Exptl Med.,* **57**: 244–248 (1944).

——— and ———: Strain Specificity and Production of Antibiotic Substance Variations and Mutations among Actinomyces, with Special Reference to *myces griseus, Proc. Nat. Acad. Sci.,* **31**: 129–137 (1945).

Schenck, J. R., and M. A. Spielman: Formation of Maltol by Degradation of St mycin, *Jour. Am. Chem. Soc.,* **67**: 2276 (1945).

Scott, E. G.: Streptomycin. A Review of the Literature, *Delaware State Med.* **18**: 18–19 (1946).

Scudi, J. V., G. E. Boxer, and V. C. Jelinek: Color Reaction Given by Strepto *Science,* **104**: 486 (1946).

Silcox, H.: Production of Streptomycin, *Chem. Eng. News,* **24**: 2763–2764 (19

Squibb, E. R., and Sons: "Streptomycin Therapy," New York, 1947.

y, A. R.: Improving Streptomycin Yields by Strain Selection and Inoculum evelopment, *Jour. Bact.*, **53**: 254 (1947).

-: Recent Progress in the Production of Streptomycin and Penicillin, paper resented before Ohio-Michigan Regional A.C.S. meeting in Toledo, Ohio, Mar. 5, 1947.

ins, R. B., and H. J. Robinson: A Method for Determination of Streptomycin Body Fluids, *Proc. Soc. Exptl. Biol. Med.*, **59**: 255–257 (1945).

E., and J. Fried: The Use of "Counter-current Distribution" for the Analysis Streptomycin Preparations, *Jour. Biol. Chem.*, **168**(1): 393–394 (1947).

olah, R. W., and G. L. Christenson: Chemical Inactivation of Streptomycin, *rch. Biochem.*, **12**: 7–12 (1947).

de la Lastra, J. L.: Streptomycin, *Med. colonial* (*Madrid*), **9**: 448–470 (1947).

nan, S. A.: Production and Activity of Streptomycin, *Jour. Bact.*, **46**: 299–310 943).

: Standardization of Streptomycin, *Science*, **102**(2637): 40–41 (1945).

: The Discovery and Development of Streptomycin, *Nucleus*, **24**(7): 171, 3 (1947).

: "Streptomycin—Nature and Practical Applications," The Williams & Wilkins mpany, Baltimore, 1949.

and H. C. Reilly: Agar-streak Method for Assaying Antibiotic Substances, *d. Eng. Chem., Anal. Ed.*, **17**: 556–558 (1945).

, ———, and A. Schatz: Strain Specificity and Production of Antibiotic Subnces. V. Strain Resistance of Bacteria to Antibiotic Substances, Especially to reptomycin, *Proc. Nat. Acad. Sci.*, **31**: 157–164 (1945).

and A. Schatz: Streptomycin—Origin, Nature and Properties, *Jour. Am. arm. Assoc., Sci. Ed.*, **34**(11): 273–291 (1945).

———, and H. C. Reilly: Metabolism and the Chemical Nature of *Streptoces griseus*, *Jour. Bact.*, **51**: 753–759 (1946).

and H. B. Woodruff, Selective Antibiotic Action of Various Substances of icrobial Origin, *Jour. Bact.*, **44**: 373–384 (1942).

r, H. A.: Penicillin and Streptomycin—A Review of Their Current Uses, *Jour. nsas Med. Soc.*, **48**: 261–273 (1947).

## SYNNEMATIN B

n, E. P., G. G. F. Newton, K. Crawford, H. S. Burton, and C. W. Hale: halosporin N, a New Type of Penicillin, *Nature*, **171**: 343 (1953).

———, J. R. Schenk, M. P. Hargie, B. H. Olson, D. M. Schuurmans, M. W. er, and S. A. Fusari: Identity of Cephalosporin N and Synnematin B, *ure*, **176**: 551 (1955).

H. S., and E. P. Abraham: Isolation of Antibiotics from A Species of *halosporium*—Cephalosporins $P_1$, $P_2$, $P_3$, $P_4$, and $P_5$, *Biochem. Jour.*, **50**: 168 1).

K., Jr., H. H. Fricke, and B. Lanius: The Purification of Synnematin B by Exchange Resins, "Antibiotics Annual 1956–1957," Medical Encyclopedia, ., New York, 1957, pp. 749–754.

ll, R. Y., J. M. Roberts, L. M. Portwood, and J. C. Jennings: Synnematin, an ibiotic Produced by *Tilachlidium*, *Proc. Soc. Exptl. Biol. Med.*, **76**: 307 1).

G. L., D. Pikula-Vrabec, J. Daly, G. Sarrocco, and T. F. Lenert: The Action ynnematin against Artificially Induced *Salmonella* Infections in Mice, "Antiics Annual 1956–1957," Medical Encyclopedia, Inc., New York, 1957, 793–808.

K., and N. Primack: Pharmacologic Studies of Synnematin B, "Antibiotics ual 1956–1957," Medical Encyclopedia, Inc., New York, 1957, pp. 755-780.

r, J. C., F. J. Kirchmeyer, and P. J. Farago: Synnematin B Blood Levels ties, "Antibiotics Annual 1956-1957," Medical Encyclopedia, Inc., New York, , pp. 781–785.

### TETRACYCLINES

Dowling, H. F.: “Tetracycline,” Medical Encyclopedia, Inc., New York,

Duggar, B. M., et al.: Aureomycin—a New Antibiotic, *Ann. N.Y. Acad* (Art. 2): 175–342 (1948).

Forbath, T. P.: Tetracycline: Engineered to Market, *Chem. Eng.,* Mar pp. 228–231.

Lepper, M. H.: “Aureomycin (Chlortetracycline),” Medical Encyclopedia, I York, 1957.

Musselman, M. M.: “Terramycin (Oxytetracycline),” Medical Encyclope New York, 1957.

Putnam, L. E., F. D. Hendricks, and H. Welch: Tetracycline, a New A *Antibiotics & Chemotherapy,* **3**: 1183 (1953).

Reeves, R. V.: Terramycin: From Dirt to Drug, *Chem. Eng.,* 1952, pp. 145-

Regna, P. P., and I. A. Solomons: The Chemical and Physical Properties o mycin, *Ann. N.Y. Acad. Sci.,* **53** (Art. 2): 229–237 (1950).

Sobin, B. A., A. C. Finlay, and I. H. Kane: U.S. Patent 2,516,080, 1950.

Waksman, S. A., and H. A. Lechevalier: “Guide to the Classification and Id tion of the Actinomycetes and Their Antibiotics,” The Williams & Wilkin pany, Baltimore, 1953.

### VANCOMYCIN

Geraci, J. E., F. R. Heilman, D. R. Nichols, W. E. Wellman, and G. T. Ross Laboratory and Clinical Experiences with a New Antibiotic, Vancomycin, biotics Annual 1956–1957,” Medical Encyclopedia, Inc., New York, 1957, 106.

Kirby, W. M. M., and C. L. Divelbiss: Vancomycin: Clinical and Lab Studies, “Antibiotics Annual 1956-1957,” Medical Encyclopedia, Inc., New 1957, pp. 107–117.

Lee, C.-C., R. C. Anderson, and K. K. Chen: Vancomycin, a New Antibiotic. tribution, Excretion, and Renal Clearance, “Antibiotics Annual 1956–1957,” cal Encyclopedia, Inc., New York, 1957, pp. 82–89.

McCormick, M. H., W. M. Stark, G. E. Pittenger, R. C. Pittenger, and McGuire: Vancomycin, a New Antibiotic, “Antibiotics Annual 1955–1956,” cal Encyclopedia, Inc., New York, 1956, pp. 606-611.

Ziegler, D. W., R. N. Wolfe, and J. M. McGuire: Vancomycin, a New Anti II. In Vitro Antibacterial Studies, “Antibiotics Annual 1955–1956,” Medic cyclopedia, Inc., New York, 1956, pp. 612–618.

## Some General References concerned with the Preservation of Foods with Antibiotics

Andersen, A. A.: Effect of Subtilin on Spores of *Clostridium botulinum, Jour.* **64**: 145–149 (1952).

Anonymous: Antibiotics in the Preservation of Foods, Data Sheet 505, Chas. & Co., Inc., Brooklyn, N.Y., June, 1955.

Campbell, L. L., Jr., and R. T. O'Brien: Antibiotics in Food Preservation, *Technol.,* **9**: 461–465 (1955). (This article contains an extensive bibliogr

Dunn, C. G.: Food Preservatives, “Antiseptics, Disinfectants, Fungicides and S zation,” 2d ed., edited by G. F. Reddish, Lea & Febiger, Philadelphia, pp. 668–706.

Durbin, C. G.: Antibiotics in Food Preservation, *Am. Jour. Public Health,* **4** 1306–1308 (1956).

Evans, F. R., and H. R. Curran: Preserving Action of Subtilin and Mild H Normal and Concentrated Milk, *Jour. Dairy Sci.,* **35**: 1101–1106 (Dece 1952).

kin. W. J., and W. H. Cathcart: Effect of Antibiotics in Retarding the Growth of *Micrococcus pyogenes* in Custard Fillings, *Food Technol.*, **6**: 224–229 (1952).

s. J. C., and H. D. Michener: Additives Accelerating Death of Spores in Moist Heat, *Jour. Agr. Food Chem.*, **2**: 298–302 (1954).

ıshall, C. L.: Can Antibiotics *Solve* Food Sterilization Problems, *Food in anada*, **13**: 25–28 (November, 1953).

## ome References concerning the Preservation of Poultry with Antibiotics

er, A. R., A. Abbey, M. A. Darken, M. C. Firman, E. F. Kline, V. F. Maturi, V. H. Miller, and S. D. Upham: Comprehensive Studies on the Use of Food rade Chlortetracycline in Poultry Processing, "Antibiotics Annual 1956–1957," ledical Encyclopedia, Inc., New York, 1957, pp. 822–830.

–, W. H. Miller, and H. P. Broquist: Aureomycin, Chlortetracycline and the ontrol of Poultry Spoilage, *Food Technol.*, **9**: 151–154 (1955).

r, W. H.: Antibiotic Introduced as Spoilage Inhibitor for Fresh Poultry, *Food ng.*, **28**(1): 43–48, 194 (1956).

er, J. V., F. Ziegler, and W. J. Stadelman: Recent Studies of Factors Affecting ie Shelf-life of Chicken Meat, *Wash. State Coll., Agr. Expt. Sta. Circ.* 254, Sep- mber, 1954.

an, H., A. Abbey, M. A. Darken, M. C. Firman, E. F. Kline, W. H. Miller, and D. Upham: An Investigation of the Process Using Food Grade Chlortetracy- ine as Applied to Turkeys, "Antibiotics Annual 1956–1957," Medical Encyclo- edia, Inc., New York, 1957, pp. 849–854.

r, F., and W. J. Stadelman: The Effect of Aureomycin Treatment on the Shelf fe of Fresh Poultry Meat, *Food Technol.*, **9**: 107–108 (1955).

## ome References concerning the Preservation of Meat with Antibiotics

rage, F. E.: The Use of Antibiotics in the Preservation of Foods Other than sh, Proceedings First International Conference on Antibiotics in Agriculture, *tl. Research Council, Natl. Acad. Sci. U.S. Publ.* 397, 1956, pp. 211–222.

rg, H. S., H. H. Weiser, and F. E. Deatherage: Studies on Meat. IV. The Use Antibiotics in the Preservation of Fresh Beef, *Food Technol.*, **7**(4): 165–166 53).

H. H., H. S. Goldberg, V. R. Cahill, L. E. Kunkle, and F. E. Deatherage: servations on Fresh Meat Processed by the Infusion of Antibiotics, *Food hnol.*, **7**(12): 495–499 (1953).

L. E. Kunkle, and F. E. Deatherage: The Use of Antibiotics in Meat Proc- ng, *Appl. Microbiol.*, **2**(2): 88–94 (1954).

## ome References concerning the Preservation of Fish with Antibiotics

. W., and H. L. A. Tarr: Effect of Chlortetracycline and Storage Tempera- es on the Quality of Shucked Oysters, *Fisheries Research Board Can., Progr. pts.*, **105**: 12–13 (1956).

L.: Antibiotics as Aids in Fish Preservation. I. Studies on Fish Fillets and imp, *Food Technol.*, **8**: 503 (1954).

M. C., A. Abbey, M. A. Darken, A. R. Kohler, and S. D. Upham: Effect of eomycin Chlortetracycline on Fish Freshness, *Food Technol.*, **10**: 381–384 6).

L. A.: Control of Bacterial Spoilage of Fish with Antibiotics, Proceedings International Conference on Antibiotics in Agriculture, *Natl. Research ncil, Natl. Acad. Sci. U.S. Publ.* 397, 1956, pp. 199–209.

. W. Boyd, and H. M. Bissett: Experimental Preservation of Fish and Beef Antibiotics, *Jour. Agr. Food Chem.*, **2**: 372–375 (1954).

3. A. Southcott, and H. M. Bissett: Effect of Several Antibiotics and Food ervatives in Retarding Bacterial Spoilage of Fish, *Fisheries Research Board , Progr. Repts.*, **83**: 35–38 (1950).

Tomiyama, T., S. Kuroki, D. Maeda, S. Hamada, and A. Honda: A Effects of Aureomycin-containing Sea Water and Ices upon the St Round Herring, *Food Technol.,* **10**(5): 215–218 (1956).

Upham, S. D., F. E. Stirn, J. F. Weidenheimer, F. M. Callahan, an Method of Dispersing Aureomycin in Ice, *Southern Fisherman Ye*

### Some References concerning the Use of Antibiotics to Improve Pla

Ark, P. A.: Streptomycin Tested for Fire Blight Control, *Western F* March, 1954.

Brian, P. W.: Antibiotics as Systemic Fungicides and Bactericides, *Ann.* **39**: 434–438 (1952).

Goodman, R. N., and M. R. Johnston: Stability of Streptomycin in Potato Tissue, "Antibiotics Annual 1956–1957," Medical Encyclopedi York, 1957, pp. 1006–1009.

Rhodes, A.: Tests with Antibiotics on Carnations, *The Grower,* **44**: 1510 24, 1955).

Stubbs, J.: The Evaluation of Systemic Fungicides by Means of *Alternari* Tomato, *Ann. Appl. Biol.,* **39**: 439–441 (1952).

Zaumeyer, W. J.: Antibiotics and Plant Health, *Jour. Agr. Food Chem.,* (February, 1955).

———: Improving Plant Health with Antibiotics, Proceedings First In Conference on the Use of Antibiotics in Agriculture, *Natl. Researc Natl. Acad. Sci. U.S. Publ.* 397, 1956, pp. 171–187.

———: Control of Powdery Mildew of Beans with Two Antibiotics, " Annual 1956–1957," Medical Encyclopedia, Inc., New York, 1957, pp.

——— and R. E. Wester: Control of Several Fungus Diseases of Beans Beans with Antibiotics, *Phytopathology,* **46**: 470 (1956). (Abstract.)

### Some General References concerning Antibiotics

Anonymous: Proceedings of the First International Conference on the Us biotics in Agriculture, *Natl. Research Council, Natl. Acad. Sci. U.S.* 1956.

Benedict, R. G.: Antibiotics Produced by Actinomycetes, *Botan. Rev.,* 19 230 (1953).

Brian, P. W.: Antibiotics Produced by Fungi, *Botan. Rev.,* **17**: 357–430 (1

——— et al.: *Brit. Mycol. Soc. Trans.,* **29**: 173–187 (1946); **32**: 30–33 (1949)

Burkholder, P. R., S. H. Sun, J. Ehrlich, and L. Anderson: Criteria of Spe the Genus Streptomyces, *Ann. N.Y. Acad. Sci.,* **60** (Art. 1): 102–123 (1

Campbell, A. H.: The Production of Antibiotics, *Research,* **6**: 42–50 ( 1953).

Day, W. H., W. C. Serjak, J. R. Stratton, and L. Stone: Contamination I Antibiotics as Contamination-control Agents in Grain Alcohol Ferm *Jour. Agr. Food Chem.,* **2**: 252–258 (1954).

Dunn, C. G.: Recent Developments in the Field of Industrial Microbio Their Possible Impact on Brewing Technology, *Am. Soc. Brewing Proc.,* 1955, pp. 65–79.

Eagle, H., and A. K. Saz: Antibiotics, *Ann. Rev. Microbiol.,* **9**: 173–226 (19

Finlay, A. C., G. L. Hobby, S. Y. P'an, P. P. Regna, J. B. Routien, D. G. M. Shull, B. A. Sobin, I. A. Solomons, J. W. Vinson, and J. H. Kan mycin, a New Antibiotic, *Science,* **111**: 85 (1950).

Fischbach, H., and J. Levine: The Identification of the Antibiotics, *Antil Chemotherapy,* 3(11): 1159–1169 (1953).

Gottlieb, D.: The Physiology of the Actinomycetes, *Istituto Superiore d* Rome, 1953.

Green, S. R., and P. P. Gray: A Differential Procedure for Bacteriologica Useful in the Fermentation Industry, *Wallerstein Labs. Communs.,* 14( 295 (1951).

ve, D. C., and W. A. Randall: "Assay Methods of Antibiotics, a Laboratory
Manual," Medical Encyclopedia, Inc., New York, 1955.

ris, D. A., and M. L. Ruger: Microbiological Aspects of New Antibiotic Screen-
ing. I. A Plug Test Procedure, *Antibiotics & Chemotherapy,* **3**: 265–270 (1953).

ley, H. B.: Dry Nisin Preparation, Canadian Patent 511,616, Apr. 5, 1955.

sar, A. E., and H. L. Holley: "Antibiotics and Antibiotic Therapy," The Mac-
millan Company, New York, 1954.

son, G. G., and M. Finland: Comparison of Methods for Determining Sensi-
tivity of Bacteria to Antibiotics in Vitro, *A.M.A. Arch. Internal Med.,* **88**: 446–
460 (1951).

s, T. H.: "Antibiotics in Nutrition," Medical Encyclopedia, Inc., New York,
1955.

e, E. F., et al.: Improved Quality of Fish Fillets as a Result of Acronize Chlor-
etracycline, Antibiotics Symposium, Washington, D.C., Oct. 17–19, 1956.

evalier, H. A., and C. T. Corke: The Replica Plate Method for Screening Anti-
iotic-producing Organisms, *Appl. Microbiol.,* **1**(2): 110–112 (1953).

K. A.: Some Applications of Biological Control Methods in the Manufacture
f Antibiotics, *Jour. Appl. Bact.,* **17**(1): 125–135 (1954).

s, E. H., A. Frisbey, R. Y. Gottshall, and J. C. Jennings: The Occurrence of
ntibacterial Substances in Seed Plants with Special Reference to *Microbacte-
ium tuberculosis,* Mich. State Univ., Agr. Exp. Sta., Quart. Bull. 37–50, Febru-
ry, 1955.

, M.: Antibiotics, *Repts. Progr. Appl. Chem.,* **37**: 677–700 (1952).

–: Antibiotics, *Repts. Progr. Appl. Chem.,* **38**: 411–436 (1953).

d, A. E., H. Raistrick, and P. Simonart: LX. Griseofulvin $C_{17}H_{17}O_6Cl$, a Meta-
olic Product of *Penicillium griseo-fulvum* Dierckx, *Biochem. Jour.,* **33**: 240
1939).

an, D.: Physiological Studies of the Actinomycetes, *Botanical Review,* **19**(1):
(1953).

R.: *Am. Jour. Bot.,* **32**(8): 528 (1945).

n, J. B., and A. C. Finlay: Problems in the Search for Microorganisms Pro-
cing Antibiotics, *Bact. Rev.,* **16**: 51–67 (1952).

r, C. E., C. W. Emmons, and H. M. Tsuchiya: "Henrici's Molds, Yeasts, and
tinomycetes," John Wiley & Sons, Inc., New York, 1947.

B. A., A. R. English, and W. D. Celmer: PA 105, a New Antibiotic, "Anti-
tics Annual 1954–1955," Medical Encyclopedia, Inc., New York, 1955, p. 827.

ing, E. H., and T. G. Anderson: Selection of Antimicrobial Agents by Labora-
y Means, *Jour. Am. Med. Assoc.,* **147**: 1336 (1951).

ov, F. B., and J. B. Bockelmann: Contamination Inhibition Antibiotics as
ibitors of Microbiological Contamination in Beer, *Jour. Agr. Food Chem.,*
0): 1219–1222 (1953).

ofler, L. A., and R. J. Hickey (eds.): "Industrial Fermentations," Chemical
blishing Company, Inc., New York, Vol. I, 1953; Vol. II, 1954.

n, P. F., H. A. Lechevalier, and S. A. Waksman: Antibiotics of Actinomycetes
h Special Reference to Their Role in the Physiology of the Organisms Pro-
cing Them, Istituto Superiore di Sanità, Rome, Sept. 7–11, 1953, pp. 147–173.

an, S. A.: *Soil Science,* **8**: 112 (1919).

"The Actinomycetes, Their Nature, Occurrence, Activities and Importance,"
ronica Botannica Co., Waltham, Mass., 1950.

"Soil Microbiology," John Wiley & Sons, Inc., New York, 1952.

The Biology of the Actinomycetes and Their Economic Importance, Istituto
eriore di Sanità, Rome, Sept. 7–11, 1953, pp. 1–12.

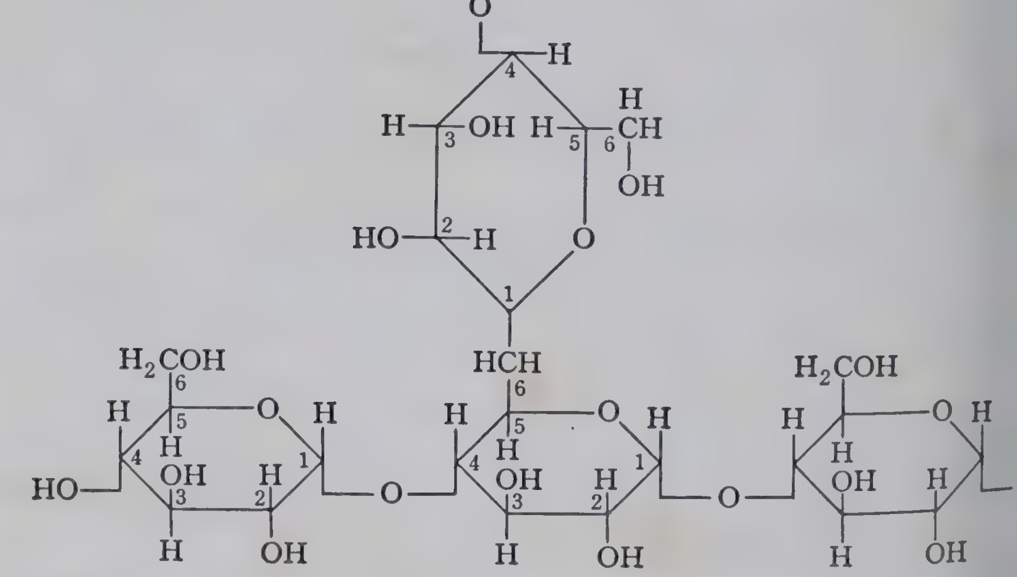
Species Concept Among the Actinomycetes with Special Reference to the
us Streptomyces, *Bact. Rev.,* **21**(1): 1–29 (1957).

## CHAPTER 46

# SACCHARIFYING AGENTS: METHODS OF PRODU AND USES

**Introduction.** Starches, hemicelluloses, and celluloses must b
lyzed or converted to fermentable sugars by enzymatic or chemic
cies before they may be used for certain industrial purposes,
ethanol production. A variety of methods may be used for co
complex carbohydrates to relatively simple materials. Some
methods are applicable in the case of starches alone, and othe
suitable modifications, may be used for the saccharification o
starches or cellulose materials.

**Nature of Starch.** Natural starches, which are polymers of
are made of two fractions: an amylose fraction, which is a linear p
and which contains 70 to 2,100 glucose units (Redfern, 1950) co
by the $\alpha$-1,4-glucosidic linkage; and an amylopectin fraction, whi
branched polymer with branches formed by the $\alpha$-1,6-glucosidic l
but with most of the glucose units joined by the $\alpha$-1,4-glucosidic l
Examples of the two types of linkages are given below.



**Methods of Saccharifying Starch-containing Materials.** In ge
the methods of saccharifying starch-containing materials involve t
of enzyme preparations or dilute acids, or a combination of the two

yme preparations that may be used include malt, which is of cereal gin, and those of microbial origin, among which are mold and bacterial ducts. Illustrative of preparations of fungal origin are mold bran and gal amylases. Either liquid or solid bacterial preparations are avail-. A method that depends upon growing an amylase-producing mold he substrate that is to be fermented after saccharification is known as "Amylo process." Dilute acids, particularly hydrochloric acid, may be l to convert grains, potatoes, and other starch-containing raw mate-. Various combinations of malt with mold bran, or other enzyme arations of microbial origin, may be employed; or combinations of with mold enzymes may be used. The method of conversion selected depend, of course, on the use intended and the availability of the olytic agents and their relative costs.

**oking the Mash.** The purpose of cooking grain mashes is to pre- them for the conversion process in which starches are saccharified rmentable sugars. During the cooking, the starches are solubilized gelatinized.

oking may be accomplished by batch or continuous processes. In operations, cooking may be done either at atmospheric pressure temperatures below the boiling point of water by methods known as sion processes" or under pressure. The continuous processes utilize temperatures, obtained by the use of high steam pressures, and short ds of time, usually less than 1 min.

*usion Process.* Some materials, such as wheat, rye, rye malt, and v malt, may be adequately cooked by infusion processes. For exam- heat may be prepared in accordance with the following method rk, Adams, Scalf, and Kolachov (1943). Ground wheat is added to water at a temperature of 100°F., while being agitated continu- The temperature of the mash is elevated to 155°F. during a period nin., held at 155°F. for 60 min., and then lowered to 152°F. At this rature a slurry of malt may be added. Conversion may be carried 145°F. for 30 min.

*h-type Pressure-cooking.* This process is carried out in tanks r, 1941) of large capacity, for example, 10,000 gal. or more. The are equipped with agitators, inlets for the water and the grain, out- eam spargers, usually a means for producing vacuum, and other ories.

ing operation, water at a temperature of 120 to 140°F. is added to k in an amount sufficient to supply 18 to 22 gal. for each bushel of other grain. Sulfuric acid is added to bring the pH of the mash to 5.6. Then the ground corn is added slowly, with agitation. ash is heated to 305 to 310°F. by steam under pressure during a of 45 min. and held for 5 to 10 min. It is cooled to 152°F., first by it down to atmospheric pressure, and second by using vacuum. comparatively large floor space occupied by the tank, the rela- ong period of processing (3 to 4 hr.), and the difficulty of obtain- orm mixing and temperature are disadvantages.

*Continuous Pressure-cooking.* The most modern method f corn and other grains is a continuous-pressure process, which oped by Unger.

The effects of variables on the efficiency of operation were Unger. The variables were (1) the temperature of cooking, (2 of cooking, (3) the particle size of the ground grain, (4) the r amount of grain to water, (5) the pH of the mash, and (6) agit found that the most important variables were temperature and that corn meal may be adequately cooked in 16 to 60 sec. at 350 in a Schutte-Koerting jet heater. He showed that grinding wa important a factor, provided that the grain was ground enou cooked properly. The optimum practical pH range was 5.4 to 5

A continuous cooker system with a capacity for 5,000 bu. of day was designed by Unger and constructed (refer to Fig. 135).

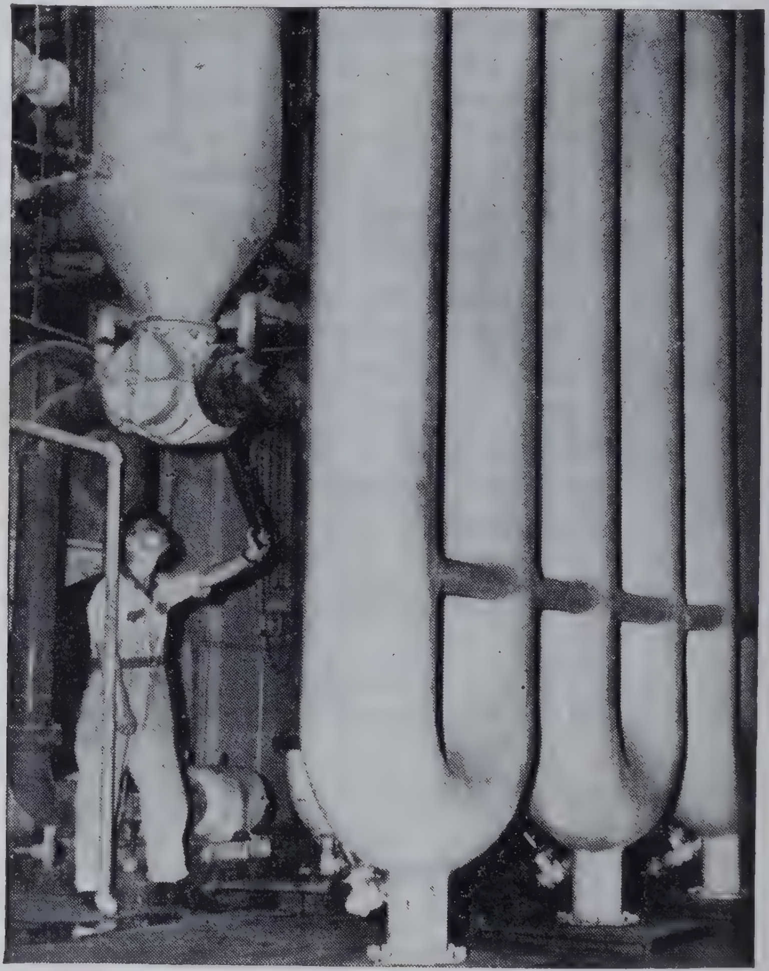


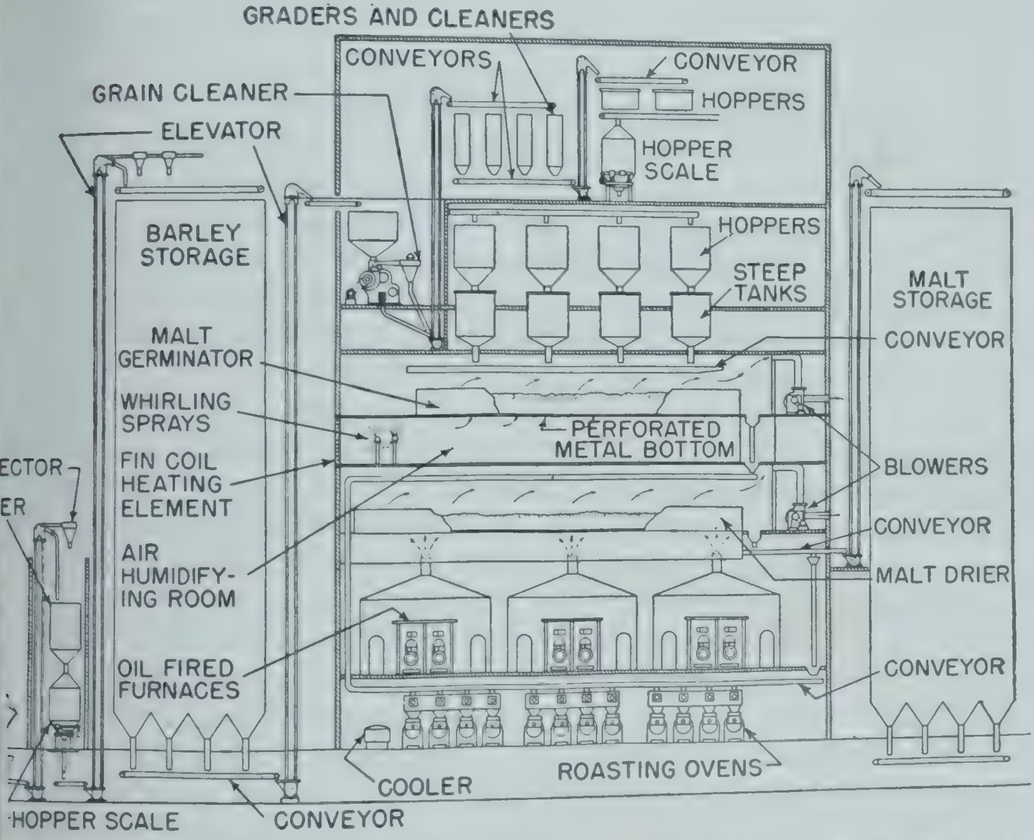
Fig. 135. Tubes for continuous cooking. The mash is heated instantaneou steam to 350–360°F. by a jet heater. It passes through a series of tubes, wh held for 60 to 70 sec. It is then cooled to 145°F. in a vacuum flash chamber. *tesy of Joseph E. Seagram & Sons, Inc., Louisville, Ky.*)

For further details, the reader is referred to other sections of thi to "Food for Thought," by Willkie and Kolachov; to Unger's thes to other sources.

## MALT

Malt is an enzymic product prepared usually from selected barley,
hough other cereal grains may be used.

**Preparation of Malt.** The manufacture of malt, commonly carried
by malsters, consists of steeping selected, screened barley, permitting
to germinate, and drying it under carefully regulated conditions. A
ematic diagram of a malting plant is shown in Fig. 136.


GRADERS AND CLEANERS
CONVEYORS
CONVEYOR
GRAIN CLEANER
HOPPERS
ELEVATOR
HOPPER SCALE
BARLEY STORAGE
HOPPERS
STEEP TANKS
MALT STORAGE
MALT GERMINATOR
CONVEYOR
WHIRLING SPRAYS
PERFORATED METAL BOTTOM
ECTOR
FIN COIL HEATING ELEMENT
BLOWERS
ER
CONVEYOR
AIR HUMIDIFYING ROOM
MALT DRIER
OIL FIRED FURNACES
CONVEYOR
ROASTING OVENS
COOLER
HOPPER SCALE
CONVEYOR

36. Malting plant (schematic diagram not drawn to scale). (*Courtesy of the*
*r, Food News, October,* 1946.)

**lection of Barley.** Barley is selected for the results that it produces.
iould be of a good variety, preferably large grained, of a fairly uni-
size, and of a light yellow color when matured. It should possess
germinating power, contain but a small amount of bitter and harsh
ances in the husk, and have the ability to produce enzymes of the
r character and quantity during germination. Soft, starchy barley
irable; damaged or weedy barley is undesirable.
rtain types of barley are sought for the manufacture of fine beer.
e United States malt is prepared principally from six-rowed barley,
igh some two-rowed barley is used in California and much of it in
pe.

**mposition of Barley.** Barley contains the four plant proteins:
in, hordein, leucosin, and edestin. Glutelin and hordein are found

mainly in the husk and aleurone cell layers; leucosin and edest endosperm between the starch granules.

Starch is found in the endosperm and is a most important co Fat, rich in lecithin, is found in the embryo and in the aleur Tannins and bitter resins are located principally in the husks. with the protein, the tannins and bitter resins are collectively re as "testinic acid."

**Storing, Cleaning, Grading.** After receipt at the malting p barley is weighed, stored, cleaned, and graded. It may be stored according to the variety. From the bins, it is conveyed to the which are of various designs and construction but which generally aspirators for blowing out light material, such as chaff, and scr separating out stones, broken barley, and other extraneous materi barley is then graded for size, each different grade going to a holding tank or hopper and being processed separately.

**Steeping.** Steeping is a process of soaking grain in order may take up sufficient moisture to quicken the living cells of the and start the processes of enzyme production and germination, precede the breakdown of cell walls and the hydrolysis of the foods.

Before the barley is steeped, it should be screened or sorted in o obtain grains of a uniform size. The moisture content can then trolled more readily during steeping.

During steeping, water is imbibed by the individual grains u optimum moisture content of 45 to 47 per cent is obtained. This tration of water favors normal germination.

Water of a known chemical composition and of a definite pH be used during the steeping process. The composition of the wat much to do with the proper removal of the tannin, bitter resin, an proteins, which are undesirable for beers. Since these substances d more readily at a higher pH, the steeping water is sometimes alkaline by the use of lime. The use of alkaline steep water appa is of advantage when malt, and subsequently beer, is made from barleys, but of no advantage when fine-skinned barleys are used.

Hypochlorites and permanganates have been used to some ext the steep water. It is claimed that they may stimulate germinatio destroy harmful microorganisms in the water. The value of their not firmly established, however.

The rate at which the water is absorbed by the grains depends variety of the barley, the size of the grain, and, mainly, the tempe of the water.

A temperature of 50 to 60°F. in the steeping tanks is satisfactory. trol of the temperature is very important.

Malting is essentially a vital process involving growth and respir Respiration rates increase with a rise in temperature and with augm water content, and oxygen is naturally required. Therefore aerati the steep water is essential. Aeration also causes foreign materia all, defective, or light barley grains to rise to the surface of the steep- tank, where they may be removed by skimming. As an alternate hod of aeration, the water may be drained off and the tank refilled.

*ndersteeping* results in a reduced rate of respiration, abnormal growth he rootlets, and an incomplete breakdown of the proteins. Less extract be secured from the final product.

*versteeping* leads to the production of a higher percentage of unger- ated grains, caused by a deficiency in the oxygen supply during ping, the inadequacy being due to the increased rate of respiration. he water is adequately aerated, the grains will germinate subse- ntly, but the development is likely to be abnormal. Hence oversteep- may lead to low yields of malt.

**ermination.** During germination several complex changes take e. The visible morphological changes include the formation of the spire, or plumule, and rootlets. A histological examination would the disappearance of the cell walls of the endosperm, while a bio- nical analysis would indicate that certain metabolic changes had n place—the breakdown of proteins, starches, and other complex ituents under the influence of enzymes. Enzymes are elaborated or ated when the temperature, moisture, and aeration conditions are factory for the germination of the seed.

e temperature, the moisture content of the grains, and the oxygen y are very important during germination. These are controlled ully in compartments or in revolving drums. Compartments are ly long and narrow. They are equipped with perforated metal ns, which allow temperature-controlled, humidified air to pass up gh and water to drain out. Conveyors or spouts distribute the d barley over the compartment floor. During the germination s, the barley is aerated with humidified air and stirred by special propellers which constantly travel back and forth from one end of mpartment to the other, and may be watered by overhead devices. mperature of the grain is maintained between 60 and 70°F. by lling the temperature of the incoming air. In rotating drums the ified and temperature-controlled air enters each drum through a of inlet tubes located near the periphery and leaves through an out- r the center. The drums revolve slowly, usually at 1 to 2 r.p.m. the germination process. Germination generally requires 5 to 7 The process is complete when the acrospire has grown to a length o three-fourths to the full length of the kernel.

oxygen supply is especially important during germination, for e of respiration increases greatly in germinating grain. During tion, heat energy is evolved, while carbon dioxide and water are d as waste respiratory products. The accumulation of carbon inhibits normal respiration, and its concentration or a deficiency gen, or both, induces abnormal respiration in which hydrogen rs other than oxygen may function. The products formed dur- ormal respiration may include acids, alcohols, and aldehydes, which exert a toxic effect on the germinating grain or young plan nating barley usually shows a respiratory coefficient of approxi

$$\text{Respiratory coefficient} = \frac{\text{Volume } CO_2 \text{ evolved}}{\text{Volume } O_2 \text{ absorbed}}$$

In the case of abnormal respiration, the respiratory coefficient i for the carbon dioxide increases, the oxygen decreases, or both occur simultaneously. For these reasons it is most important good carbon dioxide removal and to turn and properly aerate ger barley.

**Drying the Germinated Barley.** When the barley has rea desired stage of growth, drying stops the germination processes the application of heat develops color, flavor, and aroma and red moisture content of the malt. A final moisture content of appro 5 per cent is sought. Malt may then be stored without danger o down by microorganisms.

Drying is carried out in thermostatically controlled kilns or d carefully regulated temperatures. The temperatures used have do with the enzyme content of the final malt and the flavor of bee from it. Since much damage can be done to germinated barley application of too high temperatures when the grain is wet, lo peratures must be used at first. The temperature is raised gradu elevated by small increments, until a final kilning temperature o 100°C. is used.

The final temperature used depends upon the nature of th desired. For a light malt, a lower temperature should be used dark malt, a higher temperature.

During recent years, some malts have been dried in vacuum Such malts possess a high concentration of enzymes but, though factory for use in distilleries, are not so suitable for brewing, si usual protein changes are affected.

Following the kilning, the malt may be treated, usually by f to remove the radicles.

**Uses.** Malt has many uses. It is used extensively in the b industry as the chief raw material in the manufacture of beers a and as the saccharifying agent in the manufacture of industrial and distilled liquors from grains, such as wheat, corn, and rye. also used in the manufacture of malted milk, candies, cereals, an colorings but is usually roasted first in special ovens under ca controlled conditions.

**Rapid Conversion of Mash.** Gallagher and his associates (1942 described a fast method for converting grain mashes, which was ba experimental data of the following nature secured in the labor Corn cooks (mashes) were prepared under the same conditions and to 62.8°C., the temperature of conversion. Barley malt (10 per ce the form of a slurry, was added in equal amounts to each cook mixture of malt and corn was agitated mechanically, and conversio ried out for specified times. The individual mashes were cooled ickly (within 7 to 10 min.) to 22.2°C. and made up to a concentration iivalent to 38 gal. of mash per bushel of grain. The pH was adjusted 4.8 to 5.0. Then each mash was divided into four portions, three of ich were inoculated with *Saccharomyces cerevisiae* (Seagram No. 1 ain) and one of which was reserved for control purposes. Fermentation permitted to continue for 68 to 72 hr., after which the mashes were ilyzed for residual sugar and alcohol. Data based on conversion iods of 1, 5, 15, 30, and 45 min. are presented in Table 225.

LE 225. COMPARISON OF EFFECT OF MASH-CONVERSION TIMES ON FERMENTATION YIELDS *

| iveron, min. | Total sugar | | | Efficiency, per cent | | G. abs. alcohol in 100 ml. |
|---|---|---|---|---|---|---|
| | Initial g./100 ml. | Final g./100 ml. | Fermented, per cent | Fermentation | Plant basis | |
| | 11.61 | 0.627 | 94.5 | 98.4 | 93.1 | 5.52 |
| | | 0.629 | 94.5 | 99.1 | 93.8 | 5.56 |
| | | 0.630 | 94.5 | 98.4 | 93.1 | 5.52 |
| | 11.58 | 0.616 | 94.6 | 99.3 | 93.9 | 5.56 |
| | | 0.672 | 94.2 | 99.4 | 93.8 | 5.55 |
| | | 0.685 | 94.0 | 100.7 | 94.8 | 5.61 |
| | 11.54 | 0.745 | 93.5 | 100.2 | 93.5 | 5.52 |
| | | 0.715 | 93.8 | 101.1 | 94.7 | 5.59 |
| | | 0.752 | 93.8 | 100.0 | 93.4 | 5.51 |
| | 11.28 | 0.810 | 92.8 | 99.3 | 92.2 | 5.31 |
| | | 0.856 | 92.4 | 102.5 | 94.8 | 5.46 |
| | | 0.792 | 93.1 | 95.0 | 88.5 | 5.10 |
| | 11.87 | 0.856 | 92.8 | 98.0 | 90.9 | 5.52 |
| | | 0.885 | 92.5 | 89.1 | 82.3 | 5.00 |

H. Gallagher, H. R. Bilford, W. H. Stark, and P. J. Kolachov, *Ind. Eng. Chem.*, 95 (1942). Copyright 1942 by the American Chemical Society and reprinted mission of the copyright owner.

litional data obtained by Gallagher and his coworkers (1942) d that the 1-min. conversion period was equal to or better than the in. period at a pH of 5.4 or 5.9, the lower and upper limits, respec-, of levels optimum for plant conversions. More than 70 per cent of oked starch in the grain was converted to maltose by barley malt in . at 62.8°C.

rapid method for the conversion of mashes is as follows: A proning pump continuously injects a slurry of malt into a stream of mash which is being forced along by another pump. The products rough a pipe 105 ft. long and 4 in. in diameter at a temperature of . The malt acts on the grain for a period of 40 sec. The mixture cooled during a period of 1.5 min. to 21.1 to 23.9°C. This particut has a capacity for converting 5,000 bu. of grain per day.

## THE BALLS-TUCKER PROCESS

This is a process for utilizing the enzymes normally occurring i for saccharification purposes in order to save malt (Balls and 1943). Wheats contain adequate amounts of $\beta$-amylase but are ( in $\alpha$-amylase. Accordingly, it is necessary to use some malt to com for the paucity in $\alpha$-amylase.

The process is briefly as follows: A slurry of ground wheat is a to a pH of 5.2 to 5.8, and 0.05 to 0.1 per cent of $Na_2SO_3$ is added vate the inactive form of amylase and to aid in the flocculation o gluten. The mixture is agitated vigorously (for 1 hr. at 25°C. in on fication) and then permitted to stand for 45 min. or longer. The settles out, and a considerable portion of the gluten rises to the and may be recovered. A portion of the clear supernatant fluid middle layer, which contains the activated amylases, is removed an used to saccharify the cooked mash. Generally about 20 to 35 per the wheat used may be extracted with sulfite. At least 2 per cent c on the basis of the weight of the grain is necessary for saccharifi For further details, the reader is referred to the report on "The P tion of Ethyl Alcohol from Wheat" by the Northern Utilization Re and Development Division, U.S. Department of Agriculture, March

## MOLD BRAN

Mold bran is an enzyme product obtained by growing *Aspe* *oryzae* or other mold on moist, sterilized bran. It is and may be successfully as a substitute for malt in the saccharification of potato, or other types of starch-containing mashes. Smaller quan of mold bran than of malt are required to saccharify a given quant grain mash. Yields of ethyl alcohol from mashes saccharified with bran are, on the average, equal to and often superior to those obt from mashes saccharified with malt.

**Development of Process.** Takamine, in 1914, advocated the u mold enzymes (from *A. oryzae*) in the distilling industry. Studies carried out in distilleries in Canada using his mold-bran prepar (*Taka-koji*) in place of malt to saccharify grains. Although the yie alcohol obtained through the use of mold bran were reported to be h than those obtained through the use of the malt, the process wa adopted.

In 1939, Underkofler, Fulmer, and Schoene demonstrated by la tory methods that higher yields of ethyl alcohol, on the average, cou obtained from corn mashes saccharified by the use of mold bran from those saccharified by the use of barley malt. This work was confirmed by them and their associates at the Iowa State College (Sch et al., 1940; Underkofler, 1942; Hao et al., 1943; Underkofler and Fu 1943) and by Roberts, Laufer, Stewart, and Saletan (1944). Similar ings on a commercial basis have been reported by Boyer and Underk

45); Underkofler, Severson, and Goering (1946); and Underkofler, erson, Goering, and Christensen (1947). The favorable use of mold 1 for the conversion of potato mashes was reported by Beresford and istensen (1941).

**olds Used.** Hao, Fulmer, and Underkofler studied mold bran pre-d from 27 strains of molds of the genera *Aspergillus, Mucor, Peni-um,* and *Rhizopus.* They found that bran preparations made with ns of *A. oryzae, R. delemar,* and *R. oryzae* were optimum for the harification of corn mashes as evaluated by the yields of ethanol uced. Strains of *A. oryzae* were selected as being best suited for strial use, based on ease of handling, consistency of results, and high s of alcohol from the saccharified mashes. *A. oryzae* ISC 38b has used in the commercial production of mold bran.

ble 226 shows the effect of saccharification temperature and time on iol yields from corn mashes saccharified by bran preparations made selected strains of molds.

226. Effect of Saccharification Temperature and Time on Alcohol Yields from Corn Mashes Saccharified by Several Mold-bran Preparations *

| Iold used in mold-bran preparation | Saccharification | | Mold bran as per cent of corn | Alcohol yield as per cent of theoretical |
|---|---|---|---|---|
| | Temperature, °C. | Time, hr. | | |
| illus *oryzae* (A.T.C.C. No. . † | 30 | 1 | 4 | 88.6 |
| | 30 | 1 | 6 | 91.5 |
| | 30 | 1 | 8 | 91.3 |
| | 55 | 1 | 4 | 87.0 |
| | 55 | 1 | 6 | 88.4 |
| | 55 | 1 | 8 | 89.5 |
| | 55 | 2 | 4 | 86.1 |
| | 55 | 2 | 6 | 88.0 |
| | 55 | 2 | 8 | 89.5 |
| *ae* (Rohm and Haas No. | 30 | 1 | 6 | 94.5 |
| | 30 | 1 | 8 | 94.8 |
| | 55 | 3 | 6 | 93.5 |
| | 55 | 3 | 8 | 93.7 |
| *ıs oryzae* (Lockwood No. | 30 | 1 | 6 | 92.0 |
| | 30 | 1 | 8 | 93.5 |
| | 55 | 3 | 6 | 93.0 |
| | 55 | 3 | 8 | 94.0 |
| *mar* (NRRL No. 1472) ¶ | 30 | 1 | 6 | 92.5 |
| | 55 | 3 | 6 | 91.0 |

---

. Hao, E. I. Fulmer, and L. A. Underkofler, *Ind. Eng. Chem.*, 35: 814 (1943). it 1943 by the American Chemical Society and reprinted by permission of right owner.

rican Type Culture Collection, Washington.

n and Haas Co., Bristol, Pa.

Lockwood, U.S. Department of Agriculture.

Department of Agriculture, Northern Utilization Research and Development Peoria, Ill.

**Production.** Mold bran may be produced by laboratory, pil or commercial methods, some of which are described below.

*Laboratory Methods.* The present discussion will be confi drum, a pot, and a tray method for preparing mold bran.

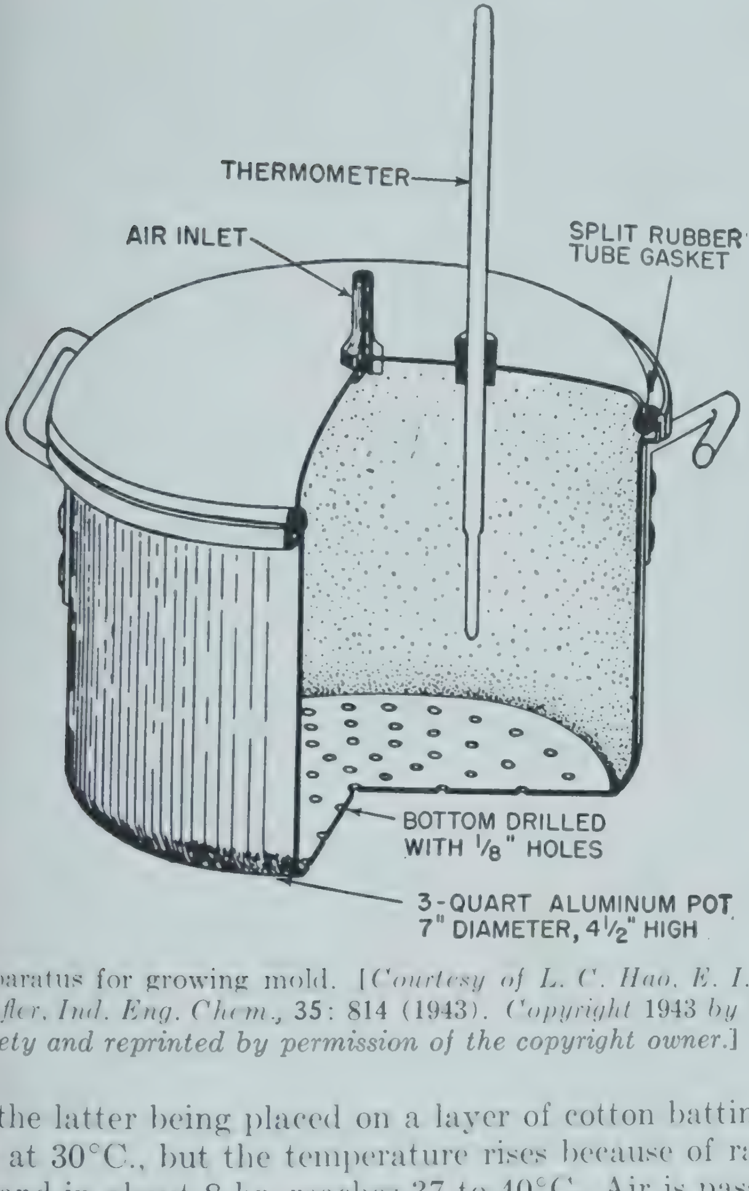
DRUM METHOD. Spore cultures are prepared in flasks to inocula for the moist, sterilized bran in drums, in accordance procedure devised by Underkofler, Severson, Goering, and Chr

Ten-g. amounts of the following medium are distributed in flasks and sterilized in the autoclave: 10 g. of ground corn, 100 g. o bran, and 60 ml. of 0.2 *N* hydrochloric acid containing 0.62 p $ZnSO_4 \cdot 7H_2O$, 0.63 p.p.m. of $FeSO_4 \cdot 7H_2O$, and 0.08 p.p.m. of CuSO After cooling, the moist, sterilized bran is inoculated with a cu *A. oryzae.* The mixture is then distributed on one side of the fla incubated at 30°C. After sporulation has reached a maximum drying of the culture is encouraged. The dry spore culture sho greenish in color, according to Underkofler and his associates, a brown or black. The ratio of the inoculum for moist, sterile bran is 1 per cent.

The well-sporulated bran culture thus prepared is used to inoc much larger charge (1,000 to 1,200 g.) of sterile bran of similar m content contained in a drum of 5-gal. capacity and capable of rotated. During operation, air is passed slowly into the drum, wl maintained at a temperature of about 30°C. preferably (never 35°C.) to favor the growth of the mold. During the germination the drum is rotated for 15 to 20 min. or less each 2 hr. Thereaft drum may be rotated continuously at not more than 1 r.p.m. until, end of 40 to 54 hr., the moldy bran is removed from the drum. It i spread thinly on a papered surface and dried at room temper Sporulation proceeds during the drying. Before being used, the product is ground in the same manner as malt.

POT METHOD. Hao, Fulmer, and Underkofler developed a labor method for the production of mold bran in 1943 which they state superior in the following respects to the drum method used earlier equipment required less space and was less complicated; the mycelium was not disturbed during growth; and uniform aeration obtained. Furthermore, the molds grew more rapidly in the pots an resultant mold bran produced relatively consistent and higher yie ethanol. The method consisted of growing selected strains of mol wheat bran mash in 3-qt. aluminum pots especially equipped for aer The apparatus, a modification of that used by Beresford and Christe is shown in Fig. 137.

A detailed description of the pot method follows: A bran mash is pared by mixing 750 g. of 0.3 *N* hydrochloric acid with 750 g. of bran. The moistened bran is packed into the aluminum pot and ster with steam at 15 lb. pressure for 30 min. The mash is cooled and inoculated with 5 to 10 g. of a well-sporulated culture of the mold ally a strain of *A. oryzae*) which has been grown on wheat bran in f as described above. The seeded bran mash is packed firmly int


THERMOMETER
AIR INLET
SPLIT RUBBER TUBE GASKET
BOTTOM DRILLED WITH 1/8" HOLES
3-QUART ALUMINUM POT 7" DIAMETER, 4½" HIGH

7. Apparatus for growing mold. [*Courtesy of L. C. Hao, E. I. Fulmer, and 'nderkofler, Ind. Eng. Chem.,* 35: 814 (1943). *Copyright* 1943 *by the American :al Society and reprinted by permission of the copyright owner.*]

' pot, the latter being placed on a layer of cotton batting. The pot bated at 30°C., but the temperature rises because of rapid growth mold and in about 8 hr. reaches 37 to 40°C. Air is passed through ss at a pressure equal to 0.3 to 3 in. of water and at such a rate e temperature is kept below 45°C. Aeration is continued for 12 r. longer, and then the moldy bran is removed from the pot, spread nly on paper, and permitted to dry at room temperature.

METHOD. The following method, described by Roberts and his rators, is employed for preparing mold bran in trays. Equal s of wheat bran and water are mixed, and the mixture is spread in 1 in. deep in aluminum trays. The moistened bran is sterilized in vs with steam at a pressure of 20 lb. for 30 min., cooled, and inocu-ith a culture of *A. oryzae*, using aseptic technique. The seeded incubated at 25°C. until the mold begins to sporulate, which ly requires about 4 days. The mold spores may be destroyed by 95 per cent ethanol to the mold-bran mixture in the proportion of l. of ethanol to 500 g. of bran and 500 g. of water. The alcoholic s removed with a hydraulic press. The resulting dried, ground ke is known as "Polidase-C."

*Pilot-plant Methods.* These are concerned principally wit
of converting laboratory procedures to plant-scale operatio
based on the report by Underkofler, Severson, Goering, and C

MIXING, STERILIZING, COOLING. These operations were acc
in a special cooker (refer to Fig. 138), which consisted of a cov

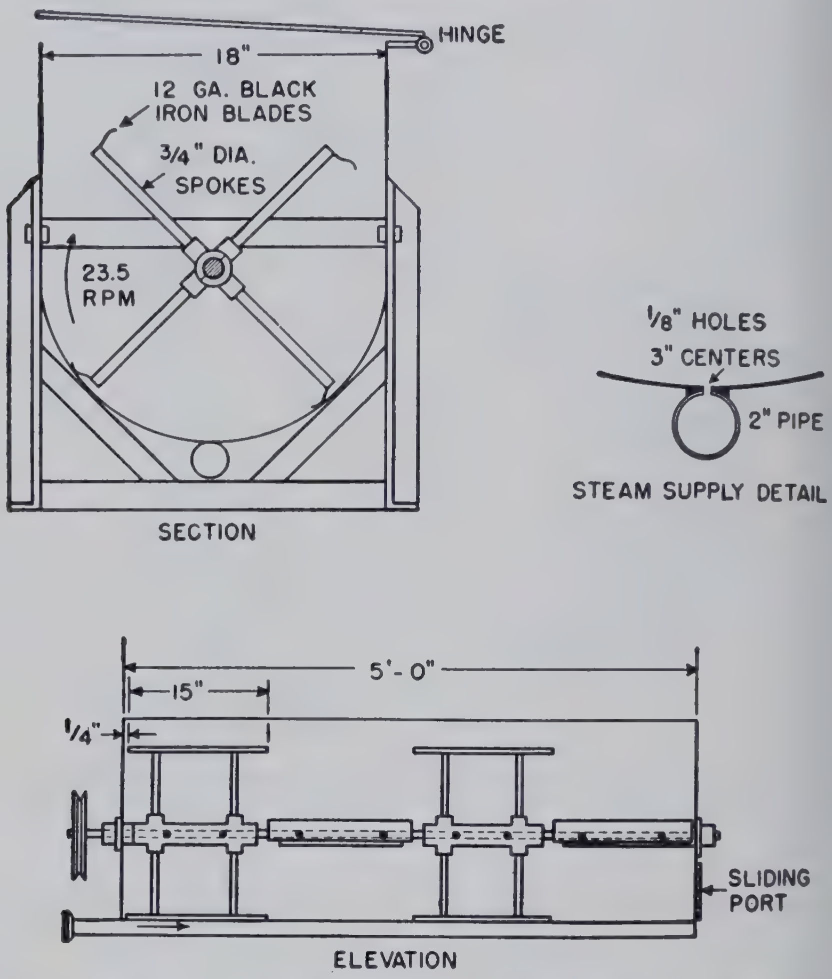


FIG. 138. Cooker detail. [*Courtesy of L. A. Underkofler, G. M. Severs*
*Goering, and L. M. Christensen, Cereal Chem.,* 24(1): 1 (1947).]

with facilities for injecting steam. The mixer was 18 in. in diam
5 ft. long. It was nondirectional and when operated at a speed of 2
agitated the bran mixture efficiently. A row of jets at the botto
cooker admitted steam for sterilization.

During operation, the bran (in 50-lb. batches) was mixed with
salt solution in the special mixer, "sterilized" by the injection o
for 30 min., and agitated continuously. A final moisture content o
51 per cent was obtained by Underkofler and his collaborators by
1 part of acid (0.2 *N* HCl) containing double the desired am
mineral salts to 2 parts of bran. Previous studies had established
that practical sterility of the acid-moistened bran could be obta
heating it at 93 to 99°C. for 15 to 30 min.; that butyric acid ba
bad type of contaminant, could be eliminated; and that the few s
mold spores caused no serious difficulties.

ooling was accomplished by blowing air through the mass, with the ker in operation, until its temperature reached about 35°C.

he moist bran was inoculated by blowing a spore culture onto the ss and continuing agitation of the mixer.

NOCULUM DEVELOPMENT. The inoculum, or spore culture, was prepared pecial galvanized iron pans, measuring 24 by 35 by 4 in. and equipped h covers containing a central air inlet and air outlets at each corner er to Fig. 139). These pans were especially designed to provide ade- te aeration of the culture, optimum kness of the bran layer, and some con- of the humidity.

ach pan received 4.5 lb. of the standard -bran-acid mixture (refer to page , which was uniformly distributed to a layer about 0.5 in. deep. The pans their contents were sterilized with n and cooled. The moist bran was inoculated with mold spores and bated at 32°C. in a constant-tempera- cabinet. Air at a rate of 1,200 to ml. per min. and at a temperature to 32°C. was admitted to each tray ugh the central inlet. When sporula- was at an optimum, usually at the of 4 or 5 days, the cover of the pan emoved and the culture was allowed -dry in the cabinet during a period hr.

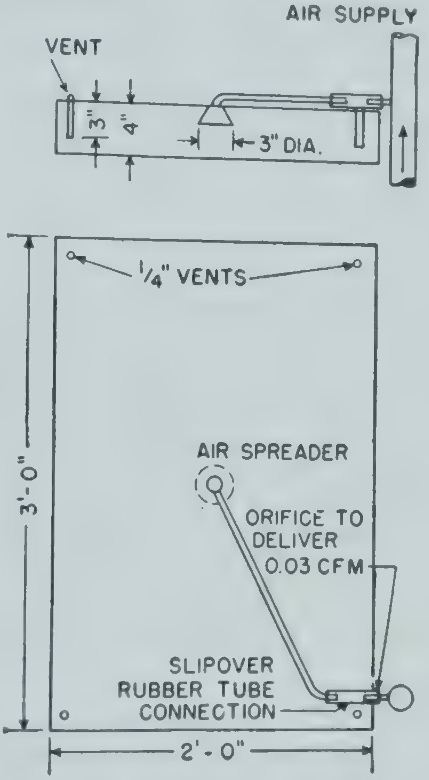


FIG. 139. Inoculum pan. [*Courtesy of L. A. Underkofler, G. M. Severson, K. J. Goering, and L. M. Christensen, Cereal Chem.*, 24(1): 1 (1947).]

CULATION METHODS. Underkofler his coworkers studied the uses of ium and of spore inocula. Mycelium a could be used, but were incon- t to handle and tended to become ontaminated for further use after r more transfers. Spore inocula l to be very satisfactory, and as as 0.04 per cent of dry spore culture could be used successfully. ver, an inoculum consisting of 0.1 per cent of dry spores was used bsequent pilot-plant studies in order to provide a reasonable factor ety against variations in spore viability.

BATION METHODS. Underkofler, Severson, Goering, and Chris- studied extensively a number of different incubation methods in arch for a pilot-plant procedure that would be rapid and practical. ound that the tray method was simple to employ but that there roblems associated with the use of the large number of trays d. In this method, it was not efficacious to pile the inoculated layers over 2 in. deep in the pans, for the mold did not grow out the mass in the deeper layers. Since the temperature of the

bran generally commenced to rise in about 5 to 6 hr. after its ino
it was necessary at this point to commence aeration of the mass
nearly saturated with moisture.

Studies were carried out using specially designed incubation c
forced aeration. Vertical and inclined cells (refer to Figs. 140 an

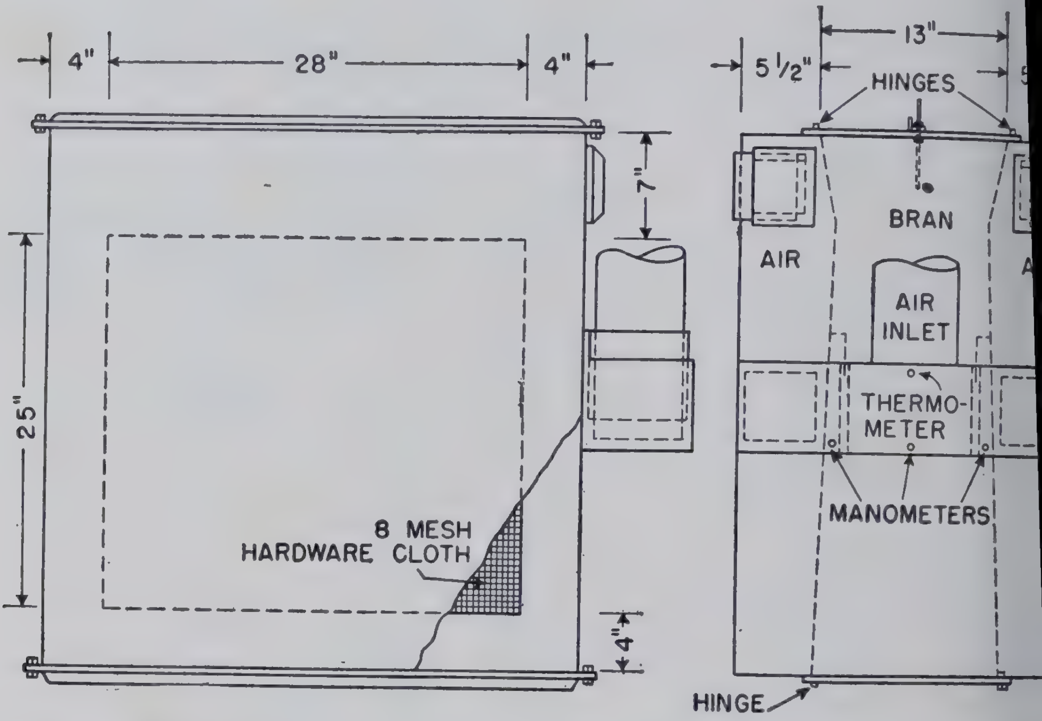
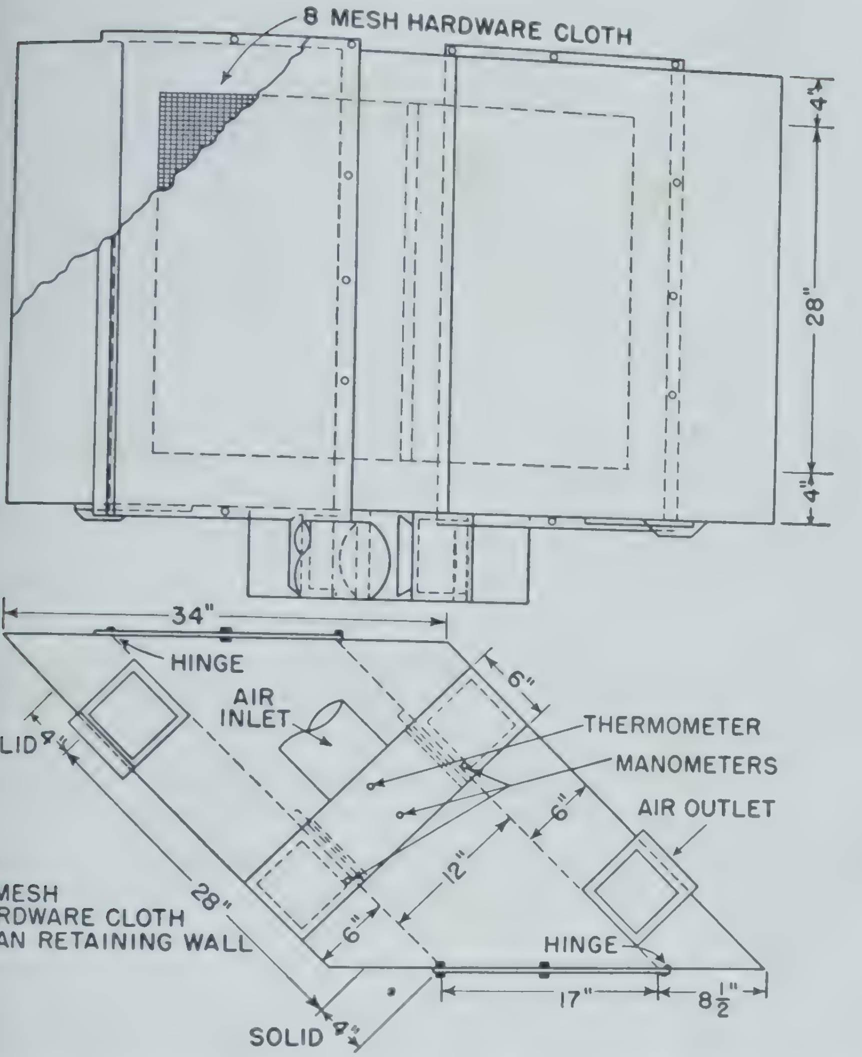


Fig. 140. Vertical incubation cell. [*Courtesy of L. A. Underkofler, G. M. Sev*
*K. J. Goering, and L. M. Christensen, Cereal Chem.*, 24(1): 1 (1947).]

equipped with hardware cloth faces, proved to be satisfactory. It
found that the temperature of incubation and the moisture content o
bran were dependent variables. The mold grew most rapidly in the
when the moisture content was adjusted suitably, between 32 and 3
The optimum moisture content resulted when 8 parts of 0.1 $N$ hydro
ric acid were used with 10 parts of bran (by weight). Under these co
tions a bran with an initial moisture content of 12 per cent conta
about 51 per cent of moisture at the time it was placed in the incub
Two-stage incubation appeared to be advantageous.

DRYING. Mold bran from the incubation cells, containing 25 t
per cent of moisture, may be dried in layers up to 4 ft. in depth in
constructed with false bottoms through which dry air is blown. Un
kofler and his associates have pointed out that the air temperatures sh
not be in excess of about 45°C. (113°F.) if serious losses in amylol
activity were to be avoided.

*Semicommercial-production Methods.* An installation for produ
mold bran on a semicommercial scale was constructed at the alcohol p
of the Farm Crops Processing Corporation (Omaha, Neb.). A cooke
similar design but somewhat larger than the one described on page
was used. Primary incubation was carried out in inclined cells; second


8 MESH HARDWARE CLOTH
4"
28"
4"
34"
HINGE
AIR
INLET
SOLID
4"
6"
THERMOMETER
MANOMETERS
6"
AIR OUTLET
12"
8 MESH
HARDWARE CLOTH
BRAN RETAINING WALL
28"
6"
HINGE
17"
$8\frac{1}{2}$"
SOLID
4"

1. Inclined incubation cell. [*Courtesy of L. A. Underkofler, G. M. Severson,*
*oering, and L. M. Christensen, Cereal Chem.*, 24(1): 1 (1947).]

tion, in closed bins containing bottoms of perforated metal. Diffi-
were encountered in connection with the usage of the primary
tion cells, for air control was found to be critical and temperature
very difficult to achieve. In addition, shrinkage of the bran
l air leakages at the top of the cells. The mold bran produced in
ıit with careful control was fair in quality.

le the first unit was in production, a successful procedure for
ıg trays was devised, which led to the construction of a new incu-
oom (refer to Fig. 142). Trays, measuring about 5 by 14 ft., were
lengthwise in such a manner that the front could be dropped down
harging mold bran and raised for filling purposes. The trays were
led in a special incubator room constructed of tile and supplied
r ducts for circulating humidified air at the proper temperature.
ondary incubator, previously used in the first unit, was employed
he mold bran. The difficulties concerned with control were absent
nit. Likewise, mold growth was rapid and the quality was uni-
good.

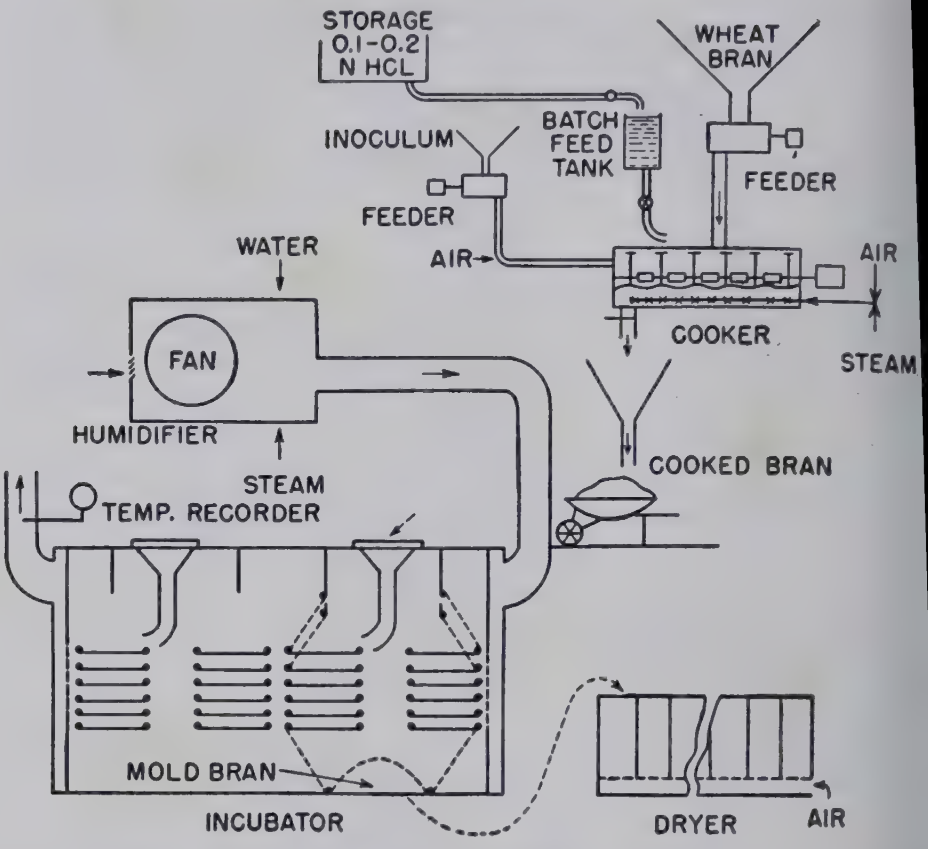

STORAGE
0.1-0.2
N HCL
WHEAT
BRAN
INOCULUM
BATCH
FEED
TANK
FEEDER
FEEDER
WATER
AIR
AIR
FAN
COOKER
STEAM
HUMIDIFIER
STEAM
COOKED BRAN
TEMP. RECORDER
MOLD BRAN
INCUBATOR
DRYER
AIR

FIG. 142. Flow sheet for semicommercial mold-bran production. [*Courtesy of Underkofler, G. M. Severson, K. J. Goering, and L. M. Christensen, Cereal C* **24**(1): 1 (1947).]

*Commercial-production Method.* Wheat bran and dilute hydroch acid (0.1 to 0.2 *N*) are mixed and heated to 100°C. with direct stea which temperature the mixture is held for 30 min. with contin agitation. The temperature of the cooked bran is reduced to 32 (90°F.) through the use of a stream of air. Spores of *A. oryzae* are oughly mixed with the bran, which contains approximately 50 per moisture and has a pH of about 3.5. The inoculated bran is distrib rather uniformly on swinging trays (arranged to facilitate easy f and removal of the mold bran) in special large-sized incubators. Hu fied air at the proper temperature is caused to flow over and unde trays until the maximum amount of mold growth has taken place, generally requires about 36 hr. The mold bran thus prepared is dro from the trays and air-dried to a moisture content of approxim 12 per cent. One commercial product is sold under the trade Eaglezyme.

**Preparation and Saccharification of Mashes.** Mashes may be pared with or without the use of acids, enzymes, and steam under p sure. A description follows of some of the methods used in the labora or on a commercial basis.

*Method of Hao, Fulmer, and Underkofler.* Sixty g. of corn meal 300 ml. of 0.04 *N* hydrochloric acid are placed in a 500-ml. Erlenm flask and thoroughly mixed. The starch of the corn is gelatinized

ng the mixture (with occasional agitation) over a low flame or on a
late.

mash is then placed in an autoclave and treated with steam at a
ure of 20 lb. per sq. in. for 30 min. The pH of the cooked and
zed mash is adjusted to 4.5 to 5.0 with sodium hydroxide, sodium
nate, or calcium carbonate. The mash, at a temperature of 30°C.,
ced in a mixer, and mold bran is added as a slurry in water. After
ion for 1 min., the mixture is returned to the 500-ml. Erlenmeyer
and incubated for 1 hr. at 30°C. for saccharification of the soluble
es. A higher temperature (55°C.) and varying times (1 to 3 hr.)
also used in some experiments for conversion. (Hao and his associ-
noculated a mash of the size mentioned above with 20 ml. of a
culture of a special strain of *S. cerevisiae* in 10 per cent malt-
t broth.)

*hod of Roberts, Laufer, Stewart, and Saletan.* Two different types
shes were used by Roberts and his associates. These are described
under the headings of pressure mashing and atmospheric mashing.

SURE MASHING. To 400 ml. of water which has been heated to
are added 97 g. of wheat and 0.5 g. of dry mold bran. The mash is
with constant stirring, at 50°C. for 15 min., and then the tempera-
raised during a 15-min. period to 66°C., at which temperature it is
or 30 min. The temperature of the mash is then raised to 93°C.
a 15-min. period and held at 93°C. for 30 min. The mash is heated
with steam for 1 hr. at a pressure of 20 lb. per sq. in. and cooled to
perature of conversion.

SPHERIC MASHING. This procedure is identical with that of pres-
ashing up to the point where the temperature reaches 66°C. The
held at 66°C. for 55 min. and cooled rapidly (within 5 min.) to
charification temperature.

arification is carried out at either 30 or 52.5°C. Mold bran
se-C) is prepared as a slurry in a concentration of 2.5 to 4 per cent
ombined weight of grain and saccharifying agent is always main-
at 100 g.—and held for 15 min. at the saccharification tempera-
he slurry is then added to the mash and saccharification effected
ing it for 30 min. at the specified saccharification temperature.

*ercial-scale Method.* Underkofler, Severson, and Goering have
l on the use of mold bran on a commercial basis at the alcohol
f the Farm Crops Processing Corporation, Omaha, Neb. The
used for preparing and saccharifying the mashes was as follows:
in is ground and prepared as a slurry, using 15 gal. of liquid per
of grain, at 43°C. (110°F.). The slurry is passed through pre-
where the temperature is raised to approximately 66°C. (150°F.)
direct injection of steam. It is then cooked for 5 min. at 166°C.
at a gauge pressure of 120 lb. The temperature of the cooked
reduced to 77°C. (170°F.) by flashing the mash through two
-reducing chambers, one at a gauge pressure of 16 lb. and the
der vacuum.

old bran is prepared with cold water as a slurry containing the correct concentration (usually 4 per cent) for saccharification slurry is mixed into a stream of hot mash, reducing the tempera mixture to 61°C. (142°F.). The mixture is held for approximat at this temperature and is then pumped through coolers to the f where it enters at a temperature of about 28°C. (82°F.). I about 3 hr. to fill a fermentor of 130,000-gal. capacity. Abou after filling has begun, yeast is pumped into the fermentors. perature is maintained at a maximum of 35°C. (95°F.) in mentor by the continuous circulation of the mash through extern Thus saccharification takes place largely in the fermentor initial pH is about 5.2 and the concentration 22.4 g. per 100 ml., of liquid per bushel of grain.

**Effect of Conditions of Saccharification on Alcohol Yiel** method used in preparing the grain mashes, the pH and concent the mashes during saccharification, the nature and concentrati mold bran used, the temperature and time of saccharification, a factors exert an important influence on the subsequent fermenta yields of alcohol obtained. Several of these factors have alrea discussed in the preceding paragraphs. Some additional informa be presented now.

Roberts and his coworkers found that maximum alcohol yie obtained with mold-bran saccharification when the mashes were at atmospheric pressure.

Naturally there is no one pH value that is optimum under al tions of saccharification. However, the use of a pH somewher range of 4.5 to 5.0 is usually suitable for saccharification, as i particularly the activities of the amylolytic enzymes, α-amyl β-amylase.

The concentration of mash at the time of saccharification upon the method used. The weight of grain usually represents a to 20 per cent of the weight of the total mash.

Hao and his associates concluded that saccharification of corn for 1 hr. at 30°C. with mold-bran preparations was as satisfac employing 1 to 3 hr. at 55°C. (Refer to Table 226.)

The usual concentration of mold bran employed for sacchari ranges from 2.5 to 4.0 per cent, depending upon the amylase act the product. A 4.0 per cent concentration based on the weight used appears to produce most satisfactory results in terms of y ethyl alcohol.

Roberts and his collaborators showed that mold-bran sacchari at 30°C. was equal to or slightly better than at 52.5°C. both in the mashes cooked at atmospheric pressure (66°C.) and in the case cooked with pressure (125°C.). They also demonstrated that t malting of mashes with barley malt increased their liquefaction not result in improving the yields of alcohol over those saccharifi only 3 to 4 per cent mold bran.

**Comparison of Mold Bran with Malt as a Saccharifying Agen** most suitable means for evaluating amylolytic agents is base ıarisons of the yields of ethyl alcohol recovered from the saccharified ıs. A number of such comparisons have been made. A few typical ts and conclusions are included below.

ıble 227 shows the results of plant-scale tests of mold bran at the

TABLE 227. RESULTS OF PLANT-SCALE TESTS OF MOLD BRAN *

| f fer-tors | Per cent of saccharifying agent in fermentor mashes | Per cent of saccharifying agent in yeast culture mashes | Av. alcohol yield/standard bu. | |
|---|---|---|---|---|
| | | | Proof, gal. | 190-proof, gal. |
| | 10 malt | 22 malt | 4.77 | 2.51 |
| | 9–10 malt | 8.6 malt + 4.3 mold bran | 5.17 | 2.72 |
| | 4 mold bran | 8.6 malt + 4.3 mold bran | 5.24 | 2.76 |
| | 9–10 malt | 8.6 malt + 4.3 mold bran | 5.15 | 2.71 |
| | 3.9–6.2 malt + 2.2–0.9 mold bran | 8.6 malt + 4.3 mold bran | 5.26 | 2.77 |
| | 9–10 malt | 8.6 malt + 4.3 mold bran | 5.23 | 2.75 |

A. Underkofler, G. M. Severson, and K. J. Goering, *Ind. Eng. Chem.*, 38: 980 Copyright 1946 by the American Chemical Society and reprinted by permis- the copyright owner.

Crops Processing Corporation, Omaha, Neb. In the tests cited, ıran was used as a replacement of supplemental malt in preparing culture mashes, with the result that the yields of alcohol were ed. This use of mold bran has resulted also in a reduction of the quired for lactic souring (i.e., acid production by lactic acid bac- ınd higher yeast counts.

e 228 shows results obtained by Roberts and his associates using ıt quantities of malt and mold bran as the saccharifying agents ı, two kinds of wheat, and two kinds of granular wheat flour. The were prepared as indicated in the previous section which was con- with the preparation and saccharification of mashes.

**omparison of Mold Preparations as Conversion Agents.** Hao mp (1945) have compared some crude, intermediate, and purified ıreparations for their conversion efficiencies and yields of ethanol ırn mash. The preparations used by them are listed in Table 229. ıshes contained, in addition to the conversion agents, 91 parts of d 1 part of liquefying material (malt), unless otherwise indicated. result of their studies, Hao and Jump made the following con- : All the crude mold-amylase preparations studied (Converzymes ı, and 511; mold brans I and II; Diatane T-1211; and Polidase-C) icient agents for converting corn mash, the conversion efficiency ıgher than that of malt but the yields of alcohol being about the he intermediate mold-amylase preparations were excellent agents

Table 228. Yield of Alcohol in Proof Gallons per 100 Pounds of Grain on Dry Basis *, †

| Saccharifying agent | Conversion temp., °C. | Corn | Winter wheat | | Spring wheat | | Winter granular wheat flour | | Spring granular | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Pressure | Pressure | Atmospheric | Pressure | Atmospheric | Pressure | Atmospheric | Pressure | Atmospheric |
| 8% malt .................. | 57.5 | 10.37 | 10.05 | 9.37 | 9.68 | 9.53 | 11.69 | 11.19 | 11.58 | 10.90 |
| 1% malt ‡ + 2% mold bran .. | 52.5 | 10.36 | 8.92 | 10.45 | 9.04 | 10.09 | 11.89 | 12.25 | 11.67 | 12.17 |
| | 30.0 | 10.34 | 9.57 | 10.35 | 9.80 | 10.10 | 11.35 | 12.39 | 11.87 | 12.42 |
| 2.5% mold bran ........... | 52.5 | 10.12 | 7.76 | 9.52 | 8.85 | 10.22 | 11.02 | 12.15 | 11.21 | 12.13 |
| | 30.0 | 10.43 | 9.58 | 9.82 | 9.38 | 10.18 | 11.19 | 12.22 | 11.66 | 12.43 |
| 3% mold bran ............. | 52.5 | 10.50 | 7.96 | 10.10 | 9.24 | 10.35 | .... | 12.42 | .... | 12.39 |
| | 30.0 | .... | .... | 10.34 | .... | .... | .... | .... | .... | 12.24 |
| 4% mold bran ............. | 52.5 | .... | .... | 10.17 | .... | 9.97 | .... | 12.40 | .... | 12.70 |
| 3% wet mold bran § ........ | 52.5 | 9.93 | .... | 10.25 | .... | 9.73 | .... | 12.47 | .... | 12.35 |
| | 30.0 | 10.24 | .... | 9.83 | .... | 9.20 | .... | 11.96 | .... | 11.68 |

---

* M. Roberts, S. Laufer, E. D. Stewart, and L. T. Saletan, *Ind. Eng. Chem.*, **36**: 811 (1944). Copyright 1944 by the American Chemical Society and reprinted by permission of the copyright owner.

† Yields are corrected for alcohol produced from malt. Alcohol from mold bran is negligible. All yields are averages of two or more results.

‡ For premalting.

§ Sample contained 50 per cent moisture; therefore, 6 g. was used.

TABLE 229. SOURCES OF MICROBIAL AMYLASE PREPARATIONS *

| Name of Sample | Producer |
|---|---|
| Bacterial amylase I | † |
| Bacterial amylase II | Wallerstein Co., Inc. |
| Bacterial amylase III | † |
| Mold bran I | Frederick Stearns & Co. |
| Mold bran II | Wallerstein Co., Inc. |
| RHozyme<br>Maltase No. 20<br>RHozyme Intermediate<br>Maltase Intermediate | Rohm & Haas Co., Inc. |
| Converzyme No. 503<br>Converzyme No. 505 | Joseph E. Seagram & Sons, Inc. (lab. preparation) |
| Converzyme No. 511 | † |
| Polidase-C<br>Polidase-S | Schwarz Lab., Inc. |
| Diatane type 1211<br>Diatane B-10 | Jeffrey Lab. |
| Taka-Diastase | Parke, Davis & Co. |
| Alase | Takamine Lab. |
| Crystazyme | Frederick Stearns & Co. |

C. Hao and J. A. Jump, *Ind. Eng. Chem.*, **37**: 521 (1945). Copyright 1945 by
ierican Chemical Society and reprinted by permission of the copyright owner.
me of producer not revealed because of limited supply of product or other
alities.

230. COMPARISON OF ALCOHOL YIELDS FROM CORN-MASH-CONVERTED PREPARATIONS AND 1 PER CENT MALT *

| Conversion material | Mash composition/ wt. ratio † | | Conversion efficiency, per cent | Yield, proof gal./ bu., based on | |
|---|---|---|---|---|---|
| | Conversion material | Liquefying material (malt) | | Corn | Total grain |
| Malt | 8 | 1 | 88.6 | 5.86 | 5.73 |
| Diatane T-1211 (control) | 5 | 1 | 90.0 | 5.93 | 5.74 |
| Diatane T-1211<br>Malt | 5<br>1 | 1 | 94.0 | 6.20 | 5.94 |
| Diatane B-10<br>Malt | 5<br>1 | 1 | 94.6 | 6.24 | 5.98 |
| Diatane B-10 | 5 | 3‡ | 96.5 | 6.37 | 6.15 |

. Hao and J. A. Jump, *Ind. Eng. Chem.*, **37**: 521 (1945). Copyright 1945 by
erican Chemical Society and reprinted by permission of the copyright

ırts corn in each case.
entrated hydrochloric acid.

for conversion and produced thinner mashes than malt and cr
preparations. The conversion efficiencies and yields of alcoho
total grain) were higher with 4 parts of RHozyme Interm
parts of Maltase Intermediate than with 8 parts of malt. T
mold-amylase preparations (RHozyme, Maltase No. 20, Ta
Polidase-S, Alase, and Crystazyme) were also excellent conver
0.25 to 1.5 parts of the agents producing satisfactory conversi

The value of mixing malt with mold-amylase preparatio
ascertained by reference to Table 230. The value of hydrochlor
liquefying agent is also indicated. (The disadvantage of usi
this purpose lies in the fact that the mash must be neutralized
conversion agent is added.)

Hao and Jump (1945) reported that the addition of the mol
preparation at intervals increased the efficiency of conversio
yield of ethyl alcohol by 1 to 2 per cent. About three-fifths of
aration was added at the conversion time and the remaining po
the fermentation had proceeded for 20 hr.

## SUBMERGED-CULTURE PRODUCTION OF MOLD AMY

Fungal amylases are produced commercially by submerge
methods. They are manufactured on the premises for use as a
stitute or supplement in the conversion of grains for such purpo
production of industrial alcohol. The amylases are generally u
out concentration or purification and may be produced on a
of media.

**Production.** The production of fungal amylases by submerge
methods has been studied by Balankura, Stewart, Scalf, an
(1946); Van Lanen and Le Mense (1946); Le Mense, Corm
Lanen, and Langlykke (1947); Adams, Balankura, Andreasen, a
(1947); Corman and Tsuchiya (1951); Pool and Underkofler
Hanson et al. (1955); and others.

Production involves growing a high-amylase-producing strain
in a suitable medium under controlled conditions of temperature,
aeration.

*Organisms Used.* Van Lanen and Le Mense and Le Mense a
ciates studied the production of amylases in a thin stillage me
367 cultures of molds, which included species of the genera *Pe*
*Aspergillus, Rhizopus, Mucor,* and *Monilia.* Only 10 per cent o
cultures of the genus *Penicillium* examined demonstrated dex
activity, and this was of low order. Cultures of the genera *R*
*Mucor,* and *Monilia* produced either very little or no dextrinizing
Greatest activity was observed in cultures of the genus *Asperg*
278 cultures studied, 34 produced the dextrinizing enzyme.
concentrations of this enzyme were produced by strains of *A.*
*oryzae, A. alliaceus, A. foetidus,* and *A. wentii.* Some strains of
produced both dextrinizing and saccharifying enzymes. Among t
*A. niger* NRRL 337 which exhibited the greatest potency of an

ures examined by the Northern Utilization Research and Develop-ıt Division group. *A. niger* NRRL 330 possesses high maltase activity. *fecia.* Various media may be used for the production of fungal lases by submerged-culture methods. Le Mense and his associates l a medium that contained thin stillage (a product of the alcoholic entation of corn and sorghums), 2 per cent of corn meal, and 0.5 per of calcium carbonate. The culture liquors obtained after growth of mold for 3 to 5 days with continuous aeration were satisfactory titutes for malt in the conversion of corn mashes.

he relative values of other media for the production of the dextriniz-nzyme by *A. niger* NRRL 337 are indicated in Table 231.

231. THE PRODUCTION OF DEXTRINIZING ENZYME BY *Aspergillus niger* NRRL 337 CULTIVATED IN VARIOUS MEDIA *, †

| Protein source | Carbohydrate source | Concentration of dextrinizing enzyme, units/ml. |
|---|---|---|
| Corn-steep liquor, 3% | None | 2.2 |
| Corn-steep liquor, 3% | Glucose, 2% | 8.2 |
| Corn-steep liquor, 3% | Molasses, 2% | 4.6 |
| Corn-steep liquor, 3% | Corn meal, 2% | 10.2 |
| Dried tankage, 2% | None | 2.1 |
| Dried tankage, 2% | Glucose, 2% | 9.3 |
| Dried tankage, 2% | Molasses, 2% | 11.5 |
| Dried tankage, 2% | Corn meal, 2% | 8.7 |
| Soybean meal, 2% | None | 7.9 |
| Soybean meal, 2% | Glucose, 2% | 7.4 |
| Soybean meal, 2% | Molasses, 2% | 8.5 |
| Soybean meal, 2% | Corn meal, 2% | 11.2 |
| Thin stillage | None | 1.7 |
| Thin stillage | Glucose, 2% | 11.5 |
| Thin stillage | Molasses, 2% | 7.9 |
| Thin stillage | Corn meal, 2% | 16.5 |
| Thin stillage | Xylose, 2% | 5.3 |
| Thin stillage | Lactose, 2% | 6.7 |
| Thin stillage | Sucrose, 2% | 11.0 |
| Thin stillage | Maltose, 2% | 14.5 |

H. Le Mense, J. Corman, J. M. Van Lanen, and A. F. Langlykke, *Jour. Bact.*, 149 (1947).

zyme determinations were made after cultures were shaken for 5 days; com-. of medium: protein and carbohydrate as shown plus 0.5 per cent of calcium te.

ns, Balankura, Andreasen, and Stark employed a medium that ıed 5 g. per 100 ml. of distillers' dried solubles (stillage from the tion column, screened to separate out most of the suspended solids, ated to about 30 per cent solids, and then dried on drum driers) . of corn meal per 100 ml. Distillers' dried solubles proved to be han stillage for mold amylase production. The pH of this medium

was adjusted to 5.0 with calcium carbonate. Adams and his coll
reported that the mold amylase produced in this medium coul
instead of barley malt and that the yield of ethyl alcohol was
0.2 to 0.3 proof gal. per bu. of grain greater than when malt was e

Hanson et al. (1955) used a fermentation medium containin
cent distillers' dried solubles and 3.2 per cent ground corn. The
adjusted to 5.5 with sodium hydroxide.

Pool and Underkofler (1953) used media containing, respective
cent distillers' dried solubles, 1 per cent corn, and 0.8 per cent
and 5 per cent distillers' dried solubles and 5 per cent corn.

*Conditions of Production.* Le Mense and his associates
50-ml. amounts of medium in 200-ml. flasks and 200-ml. am
1-liter flasks. These were sterilized with steam for 30 min. a
pressure. After cooling and inoculation, they were incubated a
and shaken continuously in a Kahn-type shaker (ninety 3-in. str
min.). Four-liter amounts of medium were used for aeration st
8-liter Pyrex cylinders, which were equipped with air spargers
perforated aluminum tubing and had aluminum plate lids. The
tainers plus their contents were sterilized for 1 hr. at 25 lb. pressu

Adams and his collaborators sterilized 1-liter portions of their
in 2-liter, cone-shaped flasks. In building up the inoculum, the
was inoculated the first time with 2.0 per cent by volume of a spo
culture (grown by surface method) of *A. niger* NRRL 337. The fla
incubated at 30°C. in a water bath, and the contents were aera
24 hr. with 0.2 cu. ft. of air per min. per liter of medium, by mean
Aloxite sparger. Thereafter the mold culture was transferred
through a number of 24-hr. submerged stages (usually two), using
cent by volume of inoculum for each succeeding mash. The fina
was aerated for 48 hr.

**Methods Used for Evaluating Submerged Fungal Amylase Pr
tions.** These are concerned with a determination of the dextrinizi
saccharifying activities of the enzymes and of the alcohol produce
grain mashes converted by them and subsequently fermented by
Adams, Balankura, Andreasen, and Stark made three determina
α-amylase activity by the 30°C. method of Sandstedt, Kneen, and
(1939); the saccharifying power by an unpublished method of
and the alcohol yields by the method of Stark and his associates (
Le Mense and his associates determined the dextrinizing activity
method of Sandstedt, Kneen, and Blish (as modified by Olson, Evan
Dickson, 1944); and the maltase activity by calculating the incre
reducing power, found by Somogyi's method (1945) after incuba
10-ml. portion of culture filtrate with 20 ml. of a 1.05 per cent m
solution for 2 hr. at 30°C. (The mixture was kept at a pH of 4.6
addition of an "acetate buffer to the maltose solution.")

**Mashing and Conversion Methods.** The method of Le Mense
was as follows: Into each of a series of 500-ml. Erlenmeyer flasks
placed 49.5 g. of ground corn, 0.5 g. of ground barley malt, and 170
tap water warmed to 70°C. The flasks were incubated in a water

0°C., and the mashes were stirred intermittently for 30 min. for preing. The mashes were then cooked for 30 min. at a steam pressure of ). per sq. in. and cooled to 75°C. The mold enzyme liquor was added ie mash with enough water to lower the temperature to 55 to 56°C., hich temperature conversion was carried out for 30 min. with frequent ition of the mashes. The same method was used for malt controls, ot that 45 g. of corn and 5 g. of malt were used, 0.5 g. of the latter g for premalting and 4.5 g. for conversion.

the procedure of Adams and his associates, the grain to be converted n) was cooked for 1 hr. at atmospheric pressure, autoclaved for ner hour at 22 lb. pressure, and cooled to the temperature of conver- Sufficient submerged mold culture was added to the mash to repre- 10 per cent of its final volume. The mash was agitated for 5 min. cooled to the temperature of setting. Ten per cent by volume of ge was then added, and the pH was adjusted to 4.8 and the total ne to 38 gal. per bu. of grain. The mash was converted at a temperaof 145°F.

**rmentation.** The corn mashes converted by the method of Le Mense oworkers were inoculated in the proportion of 2 per cent by volume a 24-hr. culture of distillers' yeast (NRRL Y567), after being cooled °C., the temperature of fermentation. The final volume in the ferution flask was about 250 ml. in each case. The fermentations were ed to continue for 72 hr. The fermented mashes (beers) were made a volume of 300 ml., of which a 200-ml. aliquot was distilled. In nstance 100 ml. of distillate was collected. The alcohol content was ined by determination of the refractive index.

**ds.** Yields of alcohol obtained by the procedure of Adams and his ates varied from 6.16 to 6.27 proof gal. per bu., as compared with roof gal. per bu. for the malt-converted control. Average alcohol of 6.05 proof gal. per 56 lb. dry corn were obtained from grain ted by the fungal amylase produced by *A. niger* NRRL 337 (Hanal., 1955).

**e Factors Affecting Amylase Production.** Erb, Wisthoff, and (1948) investigated some of the factors that influence amylase tion by *A. niger* NRRL 337. The basic medium used by them was er cent corn mash, acidified to a pH of 4.0 with sulfuric acid and l for 1 hr. at a steam pressure of 30 lb. The concentration of the n was adjusted as desired, and calcium carbonate was added to he pH to 5.3. Urea, in the proportion of 0.25 per cent, was added flask. The medium was distributed in 100-ml. amounts in 1-liter eyer flasks, sterilized, cooled, and inoculated with the spores of *r* NRRL 337 and shaken continuously at 30 to 32°C. for the period of time.

s found that the addition of small quantities of sodium chloride or um chloride (0.001 g. per 100 ml.) stimulated amylase production. me production was correlated with the concentration of the grain medium. When an incubation period of 96 hr. was used, the production of a medium with an initial concentration of 10° Brix

was decided superior to that of media of 8 and 6° Brix conc
respectively. However, based on an incubation period of 2
medium of 6° Brix concentration possessed greater enzyme act
the media of greater concentration.

Sodium pentachlorphenate (Dowicide G), in a concentratio
to 0.012 g. per 100 ml. of medium, and ammonium bifluoride,
centration of 0.015 to 0.02 g. per 100 ml. of medium, may be us
trol bacterial contamination during amylase production by
NRRL 337, according to Erb, Wisthoff, and Jacobs. Sodium pe
phenate tends to inhibit or prevent sporulation of the mold.
low amylase production and sporulation in shake-flask culture
quently correlated.

**Action of Mold Enzymes in the Saccharification of Starch.**
and Langlykke (1948) studied the action of the amylolytic
found in mold culture filtrates on the saccharification of star
action appeared to be due principally to an α-amylase and to
genic enzyme system. Little or no β-amylase was observed in t
culture filtrates. The action of the fungal α-amylase was relate
of malt α-amylase. The α-amylase liquefied starch and formed
and maltose. The glucogenic enzyme system acted upon malto
trins, and starch and formed glucose. This system differed m
from β-amylase, which produces maltose. The efficiency of sacch
tion appeared to be more closely correlated with the activity
glucogenic enzyme system than with the activity of α-amylase.

The molds used for saccharifying starches contain an α-amy
maltase (occasionally referred to as "gluc amylase," "amylogluco
or "gamma amylase"), and a limit dextrinase. The relative amo
these enzymes produced by different molds vary, as has been
strated by Pool and Underkofler (1953), who cultivated three well
organisms in submerged culture and on bran. The enzyme prepa
obtained were used to saccharify corn mashes, which were then fer
to ethanol. Cultures of *A. niger* NRRL 330 possessed the highest
activity but the lowest α-amylase activity. Mashes saccharified
tures of this mold yielded the largest amounts of ethanol. Cult
*A. niger* NRRL 337 were highest in limit dextrinase activity and
mediate, but high in respect to maltase and α-amylase activities.
saccharified with cultures of this mold yielded a little less ethan
those saccharified with *A. niger* NRRL 330. Cultures of *A. oryz*
38B, a mold used in the commercial production of mold bran, were
in α-amylase activity and lowest in maltase and limit dextrinase
ties. The alcohol yields from mashes saccharified by these culture
lowest of all. There appeared to be some correlation between
activity and alcohol yield; however, there was no correlation b
the activities of the limit dextrinase and α-amylase and the
yield.

Informative discussions concerning amylases have been present
Redfern (1950), Caldwell and Adams (1951), Schwimmer (1951
Adams (1953).

## OF BACTERIAL AMYLASE PREPARATIONS AS LIQUEFYING AGENTS

acterial amylase preparations may be used instead of malt for the ning of cooked grain mashes in the production of ethyl alcohol, rding to Hao and Jump. Table 232 compares alcohol yields from

E 232. COMPARISON OF ALCOHOL YIELDS FROM CORN MASH LIQUEFIED WITH BACTERIAL AMYLASE PREPARATIONS *

| Liquefying material | Weight ratio | | | Conversion efficiency, per cent | Yield,‡ proof gal./bu. based on | |
|---|---|---|---|---|---|---|
| | Liquefying material | Converzyme No. 505 | Corn † | | Corn | Total grain |
| Malt (control) § | 1.0 | 8 | 91 | 91.4 | 6.03 | 5.66 |
| Bacterial amylase I | 1.0 | 8 | 91 | 91.5 | 6.04 | 5.72 |
| Same | 1.5 | 8 | 91 | 92.9 | 6.14 | 5.77 |
| Same | 1.0 | 5 | 91 | 91.0 | 6.02 | 5.79 |
| Same | 0.5 | 5 | 91 | 86.8 | 5.72 | 5.53 |
| Bacterial amylase II | 1.0 | 8 | 91 | 93.7 | 6.18 | 6.00 |
| Same | 1.5 | 8 | 91 | 93.5 | 6.17 | 5.99 |
| Bacterial amylase III | 1.0 | 8 | 91 | 92.9 | 6.14 | 5.78 |
| Same | 0.5 | 8 | 91 | 91.5 | 6.04 | 5.70 |
| Conc. HCl | 3.0 ¶ | 8 | 91 | 95.5 | 6.30 | 6.10 |
| Same | 3.0 | 8 (malt) | 91 | 84.0 | 5.54 | 5.44 |

. Hao and J. A. Jump, *Ind. Eng. Chem.*, 37: 521 (1945). Copyright 1945 by erican Chemical Society and reprinted by permission of the copyright owner.

ght of corn in the mash is the same in all the fermentation runs; this corn has content of 61.8 per cent and a moisture content of 10.4 per cent.

yields are calculated on a dry basis.

malt has a starch content of 50.0 per cent and a moisture content of 8 .

acidity of the mash during cooking is approximately 0.06 N. The pH is l to 5.6 before the addition of conversion material.

ıash in which the starch liquefying agents were malt, bacterial es, and concentrated hydrochloric acid, respectively, and in which charifying agent was Converzyme No. 505. It will be noticed that ohol yields from corn mash liquefied with bacterial amylases were higher than that from the malt control when equal weights of the ing agents were used. Actually, the bacterial amylases produced of lower viscosity than the malt (based on the uses of equal of each), according to Hao and Jump. Bacterial amylase III was ed concentrate.

232 also indicates that concentrated hydrochloric acid was the ficient agent for liquefying the mash, under the conditions em- the conversion efficiency being 95.5 per cent when the saccharify- ıt was Converzyme No. 505.

## THE AMYLO PROCESS

This process is primarily one for converting starch to sugar b of selected molds, some of which have the ability to produce smal ties of alcohol from sugar.

The grain to be hydrolyzed is soaked in water for several soften it. It is then mixed with approximately twice its weight and heated at a pressure of 4 atmospheres to render the starch Acidification of the mash with 0.6 to 0.8 parts by weight of hyd or sulfuric acid per 100 parts of grain facilitates the liquefactio starch. The sterilized mash is cooled to 40 to 38°C. and inocula a pure culture of a *Mucor* or a related mold. *M. rouxii, Rhizopus icus, R. tonkinensis,* and *R. delemar* are some of the molds that ha used in the Amylo process. Sterile air is passed through the seede for 24 hr., while the temperature is maintained at 38°C. The then cooled to at least 33°C. and inoculated with yeast.

By using a modification of the Amylo process, developed by (1931), starch hydrolysis and fermentation are carried out simulta by adding at the same time *M. boulard* No. 5 and a yeast. It is that time is saved by the use of the Boulard process.

## A MODIFIED AMYLO PROCESS

The use of a modified Amylo process for the saccharification of lar wheat-flour mashes in the production of ethyl alcohol has described by Erb and Hildebrandt (1946). In brief, this process in the development of a mold inoculum, the preparation of the main m the addition of the mold inoculum and a yeast starter (*Saccharo anamensis*) to the mash, and saccharification and fermentation.

**Development of the Mold Inoculum.** Saccharification of the in the granular wheat-flour mashes is brought about by the en produced by the mold. In order to facilitate rapid saccharification mashes, it is necessary to build up a volume of inoculum equivalen to 12 per cent of the volume of the final mash. This is accomplish carrying the development through three stages, the first of which is ated in the laboratory and the next two in the plant.

*Laboratory Stage.* The mold used is a pure culture of either *Rh delemar* or *R. boulard* and is carried on slants of malt agar. The firs in the development of the inoculum is the inoculation of a special me in a 100-ml. Erlenmeyer flask with mold spores. The medium co ingredients in the following proportions: 25 g. of flour, 0.1 g. of a nium sulfate, and 500 ml. of water. It is dispensed in shallow lay 100-ml. Erlenmeyer flasks and sterilized at 250°F. (121.1°C.) for The spores produced in one flask are suspended in 3,000 ml. of wate used to inoculate the sterilized medium of the vessel employed in the plant stage described below.

*Plant Stages.* A medium containing the following materials is for 1 hr. at 250°F. (121.1°C.) in a jacketed steel pressure vessel has a working capacity of 75 gal.: 20 lb. of ground malt, 200 g. of onium sulfate, 5 g. of zinc sulfate, 100 ml. of phosphoric acid, and ient water to make 75 gal. The mash is cooled to 90°F. (32.2°C.) noculated with the 3,000-ml. suspension of mold spores prepared in aboratory. The pH of the mash before inoculation is 3.8 to 4.0. eeded mash is aerated at a pressure of 5 lb. Within a period of 20 here is abundant growth of the mold and the culture is ready for use culate the next larger medium.

the second plant stage, the medium is prepared and sterilized in a l steel pressure vessel which has a working capacity of 2,500 gal. and contains cooling coils. The medium used in this vessel is 500 gal. nular flour and malt from the mashing system of the plant, 300 gal. llage, 0.5 lb. of aluminum powder, 20 g. of zinc sulfate, 6 lb. of nium sulfate, 1,000 ml. of sulfuric acid, and sufficient water to approximately 2,500 gal. The resultant pH of the medium is 3.8 to t is sterilized at 250°F. for 1 hr. and subsequently cooled to 95°F. .). The contents of the vessel from the first stage are blown into essel to serve as its inoculum. The seeded medium is aerated at 5 lb. pressure for 24 hr.

aluminum powder is used as a constituent of the foregoing medium nteract a somewhat toxic ingredient of the stillage which appears ibit growth of the mold. The action of the interfering substance e reduced by dilution, and it may be removed by treatment with .

**paration of Main Mashes.** The mashes for conversion by malt molds are prepared in a similar manner, except for the conversion The one that is malt-converted contains 190 lb. of flour and 20 lb. t per 100 gal. Two antiseptics are added to the mash—0.75 lb. nonium bifluoride and 0.7 lb. Dowicide G for each 1,000 gal. of -for no attempt is made to sterilize it by steam. The mash is con- in a mash tub at 145°F. (62.7°C.) for 1 hr. with agitation. The hat is to be converted by the molds is prepared in the same manner, that the amount of malt is 2 per cent.

**tion of Inocula.** The control mash (malt saccharified) is cooled umped into the fermentor, and 4 per cent of yeast inoculum r), based on total charge, is added. The amount of mash and dded initially fills about 30 per cent of the fermentor. This mix- aerated for 3 to 4 hr. Then the tank is filled to 20,000 gal. by the n of two more mashes prepared as described above.

procedure with the mold inoculum is similar, except that the s of the 2,500-gal. vessel containing the mold are added to the or along with the yeast. Conversion and fermentation proceed neously.

**harification and Fermentation.** Saccharification and fermenta- complete in 40 hr. when the volume of mold inoculum represents nt of the final volume of the mash, and in 55 hr. when the volume lum represents 6 per cent of the final volume of the mash.

**Yields.** When mold was used as the principal conversion average yield from 10 fermentors was 12.1 proof gal. per 10 grain, which represented 91.2 per cent fermentation efficie malt alone was employed, the average yield from 180 ferm 11.0 proof gal. per 100 lb. of dry grain, which represented fermentation efficiency.

## ACID SACCHARIFICATION OF GRAINS AND OTHER STARCH-CONTAINING MATERIALS

Grains, vegetables, and other starch-containing materials m charified through the use of acids, such as sulfuric or hydrochl In general, the products to be converted are macerated, or grou with a measured quantity of acidulated water, and treated w under pressure.

After saccharification, the mashes may be adjusted to the d level for fermentation with ammonium hydroxide, sodium h sodium carbonate, or calcium carbonate. The use of ammoniu ide is particularly desirable, since the ammonia may act as a nitrogen for yeast growth. If sulfuric acid is used for the conv the starch and subsequently neutralized with calcium carbo precipitated calcium sulfate may be separated from the wort mentation and/or filtration.

Severson (1937) has investigated the saccharification of vari mashes with hydrochloric acid. Maximum yields of ethanol from saccharified grains averaged somewhat lower than those obtained saccharification. Highest yields of ethanol were obtained fr when the mash was heated for 2 to 3 hr. at a steam pressure per sq. in. in the presence of 0.10 *N* hydrochloric acid (Schoen 1940).

Schoene, Fulmer, and Underkofler (1940) have shown that yields of ethanol may be obtained by the addition of mold bran saccharified mashes. Their procedure for preparing acid-sac mashes, based on the optimum conditions found by Severson, follows: Measured quantities of corn meal and 0.1 *N* hydrochlo 100 g. and 500 ml., respectively, were placed in each 1,000-ml meyer flask used, and the mixture was cooked for 2.5 hr. at a stea sure of 25 lb. per sq. in. The pH of the cooked mashes was adjus with concentrated ammonium hydroxide. Weighed portions of lytic (enzyme-containing) materials were added to the acid-sac mashes, and final saccharification was carried out at 55°C. for Results of typical experiments by Schoene, Fulmer, and Underk shown in Table 233.

An examination of Table 233 indicates that the best resul obtained with mold bran. Schoene and his associates suggested increased yields resulting from the use of mold bran were due to t variety of enzymes that it contained. For example, mold bran ılsin, an enzyme that may hydrolyze gentiobiose (a disaccharide that · be formed during acid saccharification) to dextrose.

E 233. EFFECT OF MALT, SOYBEAN MEAL, AND MOLD BRAN UPON THE ALCOHOL YIELDS FROM ACID-SACCHARIFIED CORN MASH *

| alt and soybean meal | | Mold bran and malt | | | Mold bran and soybean meal | | |
|---|---|---|---|---|---|---|---|
| Proportion of material as per cent of corn | Yield, per cent | Proportion of material as per cent of corn | | Yield, per cent | Proportion of material as per cent of corn | | Yield, per cent |
| S | | MB | M | | MB | S | |
| 0 | 76.8 | 0 | 0 | 75.3 | 0 | 0 | 84.0 |
| 0 | 76.4 | 2 | 0 | 82.5 | 1 | 0 | 87.1 |
| 0 | 76.0 | 3 | 0 | 82.9 | 2 | 0 | 88.6 |
| 0 | 75.5 | 5 | 0 | 82.9 | 3 | 0 | 89.8 |
| 2 | 75.7 | 0 | 2 | 78.0 | 5 | 0 | 92.4 |
| 3 | 78.5 | 2 | 1 | 83.0 | 0 | 3 | 87.8 |
| 5 | 77.0 | 3 | 1 | 82.2 | 1 | 1 | 90.9 |
| 1 | 78.5 | 5 | 1 | 82.5 | 2 | 1 | 90.5 |
| 2 | 78.3 | | | | 3 | 1 | 90.7 |
| 3 | 76.4 | 2 | 2 | 81.2 | | | |
| 1 | 76.5 | 3 | 2 | 82.6 | 1 | 2 | 90.7 |
| 2 | 77.5 | 5 | 2 | 82.7 | 2 | 2 | 91.2 |
| 3 | 76.6 | .. | .. | ... | 3 | 2 | 91.3 |

Schoene, E. I. Fulmer, and L. A. Underkofler, *Ind. Eng. Chem.*, 32: 544 (1940). ight 1940 by the American Chemical Society and reprinted by permission of pyright owner.

xtures of mold bran and soybean meal also produced increased of ethanol from acid-saccharified corn mashes.

ording to Hayek and Shriner (1944), starch-containing mashes may drolyzed (saccharified) satisfactorily by the use of sulfurous acid. showed that pure starch may be completely converted to glucose in n. at 165°C. in the presence of 0.2 to 0.4 per cent of sulfur dioxide, orn mash may be hydrolyzed in 15 min. at 160°C. with 2 per cent of dioxide, and that wheat mash may be hydrolyzed in 10 min. at o 170°C. with 2 per cent of sulfur dioxide. As high as 95 per cent cose is obtained from corn and wheat mashes by hydrolysis with dioxide. The sulfur dioxide may be removed from the hydrolyzed s and fermentation initiated. Yields of ethyl alcohol obtained from ous acid-hydrolyzed mashes compare favorably with those secured nalt-converted mashes, according to Hayek and Shriner.

pH of a mash saccharified with acid must be adjusted before it is ated with yeast or other fermentation organism. Such adjustment pH usually increases the salt concentration of the mash.

## ACID SACCHARIFICATION OF AGRICULTURAL RESI

Dunning and Lathrop (1945), of the Northern Utilization and Development Division, reported on their research conce laboratory-scale saccharification of agricultural residues, such cobs, cottonseed hulls, flax shives, oat hulls, and sugar-cane baga

The agricultural residues, typical analyses for which are Table 234, are hydrolyzed in two stages. During the first s

TABLE 234. PERCENTAGE ANALYSIS OF REPRESENTATIVE HARDWOODS, SOFT AND AGRICULTURAL RESIDUES *

| Material | Pentosan | Cellulose |
|---|---|---|
| Ponderosa pine † | 7.4 | 52.3 |
| Tanbark oak † | 19.6 | 44.8 |
| Corncobs | 28.1 | 36.5 |
| Bagasse | 20.4 | 41.3 |
| Oat hulls | 29.5 | 33.7 |
| Cottonseed hulls | 21.0 | 35.1 |
| Flax shives | 23.0 | 38.0 |

* J. W. Dunning and E. C. Lathrop, *Ind. Eng. Chem.*, **37**(1): 24 (1945). C 1945 by the American Chemical Society and reprinted by permission of the c owner.

† G. J. Ritter and L. E. Fleck, *Ind. Eng. Chem.*, **14**: 1050 (1922). Copyright the American Chemical Society and reprinted by permission of the copyrigh

pentosans are hydrolyzed by the use of dilute sulfuric acid, and the second stage the cellulose is hydrolyzed by the use of concen sulfuric acid.

**Pentosan Hydrolysis.** The purpose of this hydrolysis is to se the pentosans as xylose and furfural without saccharifying the cel

A study of the effects of acid concentration, temperature, tim other factors upon the hydrolysis of pentosans has been made by Du and Lathrop, and it has been found that satisfactory hydrolysis m accomplished under carefully controlled conditions. For example, corncobs were treated with 1.9 per cent of sulfuric acid at a tempe

TABLE 235. EFFECTS OF ACID CONCENTRATION, TEMPERATURE, AND TIME UPON SEP OF PENTOSANS FROM HEXOSANS *

| | | | |
|---|---|---|---|
| Acid concentration (sulfuric), per cent | 1.9 | 4.4 | |
| Temperature, °C. | 121 | 100 | |
| Time, min. | 50 | 55 | |
| Solids/liquid ratio, g./100 ml. | 30:100 | 6:100 | 6: |
| 1. Yield of xylose, per cent | 86.1 | 84.1 | |
| 2. Yield of furfural (as xylose), per cent | 9.3 | 10.9 | |
| 3. Sum of 1 and 2 | 95.4 | 95.0 | |
| 4. Yield of dextrose, per cent | 0.78 | 3.2 | |

* J. W. Dunning and E. C. Lathrop, *Ind. Eng. Chem.*, 37(1): 24 (1945). Cop 1945 by the American Chemical Society and reprinted by permission of the cop owner.

21°C. for 50 min., using a solid/liquid ratio of 30:100, the yield of ›se was 86.1 per cent, that of furfural (as xylose) 9.3 per cent, and that extrose 0.78 per cent. Thus 95.4 per cent of the pentosans was hydro- d. whereas less than 1 per cent of dextrose was produced. Data erning other conditions found satisfactory for the separation of osans from hexosans may be found in Table 235.

xperiments were carried out in a five-cell, glass diffusion battery on a ratory scale to determine the value of countercurrent extraction of entosans from corncob residues. Each of the cells of the battery was pped with the required inlets and outlets and a heating element. te sulfuric acid at temperatures just under 100°C. was circulated ıgh the crushed corncobs under a variety of conditions.

a result of these experiments, certain facts become clear. Extrac- of 90 to 95 per cent of the pentosans as hydrolyzate products could be nplished consistently in 80 to 120 min. The hydrolyzates contained › 20 per cent of reducing sugar. Sharp fractionation of pentosans hexosans resulted only when conditions were limited. However, high s of xylose could be obtained under a variety of conditions.

**llulose Hydrolysis.** The cellulose in the extracted corncobs is olyzed in dilute acid solution after impregnation with concentrated ric acid. One process developed by Dunning and Lathrop was essen- as follows: The extracted corncobs were dried by filtration, by tering devices (to 50 to 55 per cent), and by the use of hot air at eratures from 60 to 70°C., until the original acid from the pentosan lysis was concentrated to 72 per cent. Residues dried in the pres-

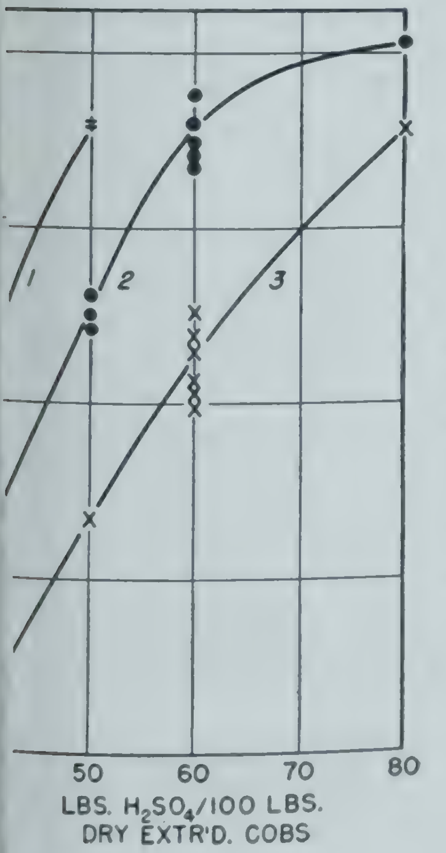


FIG. 143. Effect of variables on yield of dextrose from extracted corncobs impregnated with 85 per cent sulfuric acid at 40°C. [*Courtesy of J. W. Dunning and E. C. Lathrop, Ind. Eng. Chem.,* 37: 24 (1945). *Copyright* 1945 *by the American Chemical Society and reprinted by permission of the copyright owner.*]

ence of acid were more readily saccharified than those freed before drying. The dried material was passed through a hamm reduce it to the desired mesh size.

The dried residue was impregnated with sulfuric acid of 8 concentration by means of a machine constructed of mild st contained a hopper for feeding purposes, a discontinuous screw cooled with water, and a water-jacketed casing. Figure 143 effect of variables on the yield of dextrose from extracted impregnated with 85 per cent sulfuric acid at 40°C. Curve figure shows that a 90 per cent yield of dextrose was obtai 100 lb. of extracted corncobs (of 50-mesh size and with the acid the residue) was treated with slightly under 50 lb. of 85 per cen acid at 40°C. for 2 min. According to the authors, this quantit furic acid is considerably less than that suggested in some other fication processes involving the use of concentrated sulfuric acid.

The residue, impregnated with 85 per cent sulfuric acid, occu a dry powder, is diluted with water and permitted to undergo hy Dunning and Lathrop have reported that under optimum cond hydrolysis in 8 per cent sulfuric acid for 7 to 10 min. at tempera 120 to 130°C.—hydrolyzates were secured that contained 10.5 dextrose.

**Flow Sheet of Process.** Figure 144 presents a flow sheet of th ess developed by Dunning and Lathrop (1945).

**Products.** Table 236 shows the products that may be obtaine 1 ton of agricultural residues.

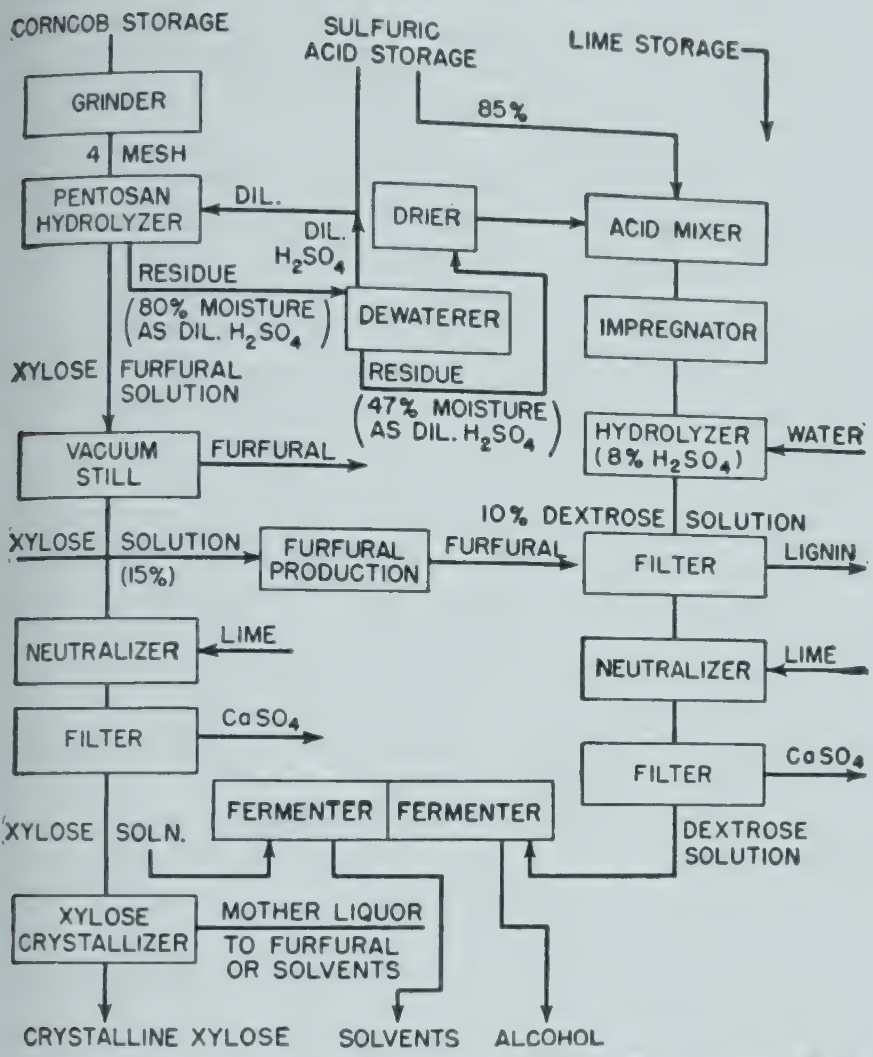
TABLE 236. PRODUCTS OBTAINABLE FROM 1 TON (DRY WEIGHT) OF AGRICUL RESIDUES *

| Agricultural residue | 93 per cent pure crystalline xylose, lb. | Furfural, lb. | 95 per cent alcohol, gal. |
|---|---|---|---|
| Corncobs .................. | 135 | 214 | 44 |
| Bagasse .................. | 98 | 155 | 49 |
| Oat hulls .................. | 141 | 224 | 42 |
| Cottonseed hulls .......... | 101 | 160 | 42 |
| Flax shives ............... | 111 | 175 | 46 |

* J. W. Dunning and E. C. Lathrop, *Ind. Eng. Chem.*, **37**: 24 (1945). Co 1945 by the American Chemical Society and reprinted by permission of the right owner.

**Uses of the Hydrolyzates.** The dextrose solution obtained hydrolysis of cellulose may be used for the production of ethyl after the lignin has been removed by filtration and the excess acidit been neutralized with lime.

The xylose solution resultant from the initial (pentosan) hydr of the residues may be used for fermentation, for furfural manufa or for the production of crystalline xylose.


CORNCOB STORAGE
SULFURIC ACID STORAGE
LIME STORAGE
GRINDER
85%
4 MESH
PENTOSAN HYDROLYZER
DIL.
DIL. $H_2SO_4$
DRIER
ACID MIXER
RESIDUE
(80% MOISTURE AS DIL. $H_2SO_4$)
DEWATERER
IMPREGNATOR
XYLOSE FURFURAL SOLUTION
RESIDUE
(47% MOISTURE AS DIL. $H_2SO_4$)
VACUUM STILL
FURFURAL
HYDROLYZER (8% $H_2SO_4$)
WATER
10% DEXTROSE SOLUTION
XYLOSE SOLUTION (15%)
FURFURAL PRODUCTION
FURFURAL
FILTER
LIGNIN
NEUTRALIZER
LIME
NEUTRALIZER
LIME
FILTER
$CaSO_4$
FILTER
$CaSO_4$
FERMENTER
FERMENTER
XYLOSE SOLN.
DEXTROSE SOLUTION
XYLOSE CRYSTALLIZER
MOTHER LIQUOR TO FURFURAL OR SOLVENTS
CRYSTALLINE XYLOSE
SOLVENTS
ALCOHOL

44. Flow sheet of the process. [*Courtesy of J. W. Dunning and E. C. Lathrop, ng. Chem.*, 37: 24 (1945). *Copyright* 1945 *by the American Chemical Society printed by permission of the copyright owner.*]

.or to its use for fermentation purposes, it is necessary to neutralize ylose solution (that obtained by the use of the countercurrent lysis method containing approximately 15 per cent xylose, 0.3 per lextrose, 1.75 per cent furfural, 2.0 per cent acetic acid, and 4.4 per sulfuric acid) and to remove the furfural. The latter objective may complished, according to Dunning and Lathrop, by feeding the hot e solution into a vacuum column and removing the furfural-water rope. The xylose may be fermented to butanol and acetone by *idium acetobutylicum* (refer to the chapter on this fermentation) 2,3-butanediol by *Aerobacter aerogenes* (refer to the chapter con- g this subject).

## ACID SACCHARIFICATION OF WOOD

many years scientists have been engaged in developing and improv- ethods and equipment for hydrolyzing wood and other cellulose- ning substances to sugars. Their efforts have been rewarded with success, for there are several processes, some fundamentally di which sugars may now be prepared on a large scale from such

1. **Historical.** Dangiville, in 1880, proposed the use of gase chloric acid to hydrolyze wood.

In Germany, Claassen (1900) developed a method for hy vegetable fibers, using sulfur dioxide or sulfuric acid. Attempts lish commercial processes in the United States, based on his resulted in failure.

In 1910, Ewen and Tomlinson (Jacobs and Newton, 1938) co the first plant for producing ethyl alcohol from sawmill waste in town, S.C. The average yield of alcohol, using dilute sulfuric a high pressure to hydrolyze the sawdust, was approximately 20 ton, although higher yields were occasionally obtained.

Willstätter (1913) demonstrated that a 40 per cent solution o chloric acid was very much different in action toward cellulose common 35 to 36 per cent commercial hydrochloric acid solutio stätter, with Zechmeister, discovered that cellulose is transform out waste to glucose within the space of a few hours by a 40 hydrochloric acid solution at room temperature.

Hägglund, Bergius, and others commenced work in 1916 whic the development of the Rheinau process.

During World War I, the Germans produced sugars from w the so-called "Stettin war process." This process corresponded process used by Ewen and Tomlinson in the United States. August, 1918, to September, 1919, the plant at Stettin (Scholler produced 300 tons of sugar and converted it to 150,000 liters o alcohol. Owing to the low yields, only about 6 liters of alcohol per of dry wood substance, it was economically unsound to continue tion of the plant after the war was over. It was later shown that t yields had been due to a partial destruction of the sugars dur hydrolytic process.

At Geneva, Terrisse and Lévy in 1920 used a combination of cent hydrochloric acid solution and gaseous hydrochloric acid to lyze wood in a method that later became known as the "Prodor p (Ormandy, 1926).

Much research concerning the production of ethyl alcohol fron wastes has been carried out by the U.S. Forest Products Labora Madison, Wis. Sherrard and Kressman (1944) reviewed the develo of wood saccharification processes in the United States from the t their inception to just before World War II. Harris, Beglinger, and Sherrard (1945) studied the hydrolysis of wood with dilute s acid in a stationary digester. They found that yields of about 50 p of reducing sugar (75 to 80 per cent fermentable) could be obtaine softwoods and yields of about 50 per cent of reducing sugar (50 to cent fermentable), from hardwoods.

The use of the rotary digester in wood saccharification was in gated by Plow, Saeman, Turner, and Sherrard (1945). They re that there were some serious disadvantages and little advantage e of the rotary digester in multistage saccharification of wood as com- red with the vertical, stationary percolator.

Saeman (1945) studied the kinetics of the hydrolysis of wood and of e decomposition of sugars in dilute acid at high temperatures. He found at the hydrolysis of hemicellulose took place easily and required only a action of the time needed for the hydrolysis of the resistant portion of lulose. Saccharification of cellulose involved two reactions: the drolysis to reducing sugar and the consecutive decomposition of the gars. The decomposition of all the sugars appeared to follow the laws a first-order reaction. An increase in acid concentration (0.4 to 1.6) temperature (170 to 190°C.) resulted in increased efficiency in the mation of reducing sugar from cellulose.

An analytical procedure, stated to be rapid, was developed for the ntitative saccharification of wood and cellulose by Saeman, Bubl, and rris (1945). The material for analysis is ground sufficiently finely to s through a 30-mesh screen. It is treated with 72 per cent sulfuric acid 30°C. for 45 min. after air-drying to a low moisture content. The mix- is diluted with water and treated for 1 hr. at a steam pressure of b., or by boiling for 4.5 hr.

rocedures for analyzing wood sugars have been described by Saeman, ris, and Kline (1945). These included an electrometric titration hod and a micro method developed by Shaffer and Somogyi for deter- ing the reducing sugars and a test for fermentable sugars.

able 237 summarizes some data concerning different methods for pro- ng ethanol from cellulose.

237. Production of Ethanol from Cellulose by Different Processes *

| Process | Year | Hydrolytic agent | Concentration, per cent | Temperature, °C. | Liters of ethanol/100 kg. of dried wood |
|---|---|---|---|---|---|
| mot | 1819 | $H_2SO_4$ | 100 | | |
| ld | 1854 | $H_2SO_4$ | 100 | | |
| sen | 1894 | $H_2SO_4$ | 0.5 | 180 | 7.6 |
| en | 1900 | $SO_2$ | ..... | 150 | 7.5 |
| and Tomlinson | 1909 | $SO_2$ | ..... | 150 | 8.0 |
| | 1912 | HCl and vapor | ..... | 140 | 9.0 |
| itter | 1913 | HCl | 40 | 20 | |
| nd | 1914 | $H_2SO_4$ | 0.4 | 175 | 8.8 |
| an | 1915 | $H_2SO_4$ | 2.5 | 174 | 10.7 |
| r | 1922 | $H_2SO^4$ | ..... | 170 | 15–20 |
| r | 1929 | $H_2SO_4$ | 0.5 | 180 | 22 24 |
| | 1931 | HCl | 40 | 20 | 32–35 |
| i-Leone | 1939 | $H_2SO_4$ | ..... | ... | 30 |

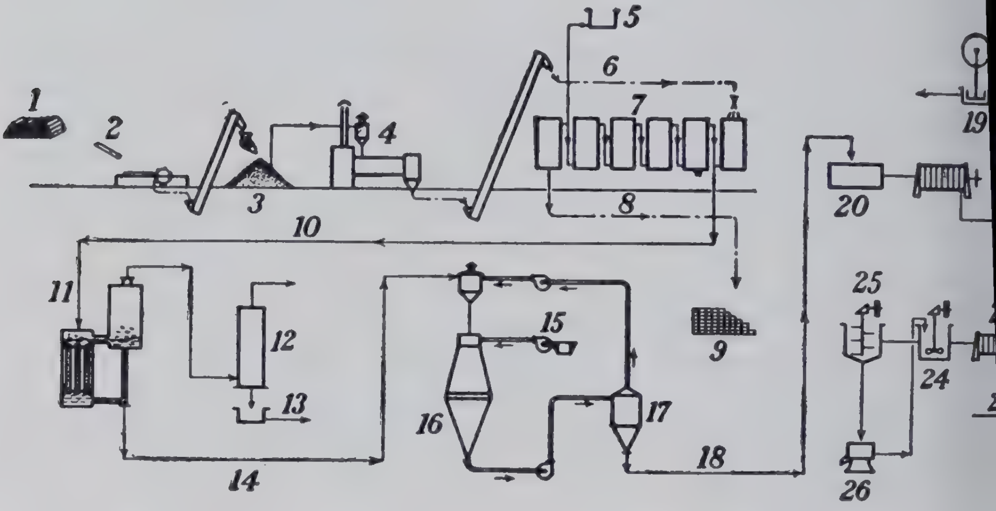
---

* Giordani, *Chimica e industria (Milan)*, 21: 265 (1939).

**2. The Bergius-Rheinau Process.** The Bergius (1937) based on Willstätter's discovery that a 40 per cent solution chloric acid will hydrolyze cellulose to glucose at room tempera

Cellulose is the principal constituent of wood. Hence a 4 hydrochloric acid solution dissolves most of the wood except

In the Bergius process, the wood is shredded and then dried ing drums to a water content of approximately 0.5 per cent. wood is next conveyed to a battery of diffusers, where hydroch acts upon it to convert it to water-soluble sugars and other end A countercurrent principle is employed in the extraction. The gresses through the battery (Fig. 145) and comes into contact



1. Wood supply
2. Wood shredder
3. Chips
4. Wood drier

Disintegration steps:
5. Hydrochloric acid
6. Dried wood chips
7. Diffusion battery
8. Lignin
9. Lignin-briquetting process

Evaporation steps:
10. Sugar solution
11. Evaporator for hydrochloric acid
12. Condenser
13. Hydrochloric acid
14. Sirup

Spraying steps:
15. Air heater
16. Spray drier
17. Cyclone separator
18. Crude sugars (raw product)

Crystallization steps:
19. Vacuum pump
20. Inversion
21. Evaporator
22. First crystallization
23. Filter press
24. Solution vat
25. Recrystallization
26. Centrifuge for pure crystals
27. Sirup filtrate (mother liquor)
28. To alcohol (by fermentation)

FIG. 145. Flow diagram of the Bergius process. [*Courtesy of F. Bergius,* *Chem.,* 29: 247 (1937). *Copyright* 1937 *by the American Chemical Societ* *printed by permission of the copyright owner.*]

wood in the first diffuser. During the extraction process, about tw of the wood by weight is dissolved, while one-third remains as lign resultant acid solution contains approximately 32 per cent by vo reducing sugar.

The acid solution containing the reducing sugars (the hydr is now distilled at 36°C. under vacuum in order to separate the the acid from the sugar. This procedure removes about 80 per the acid from the hydrolyzate. This acid is regenerated, reconce and used over again.

The hydrolyzate is further concentrated in a spray-drying chamber, iere water and hydrochloric acid are lost by evaporation. The resultant id product, which is voluminous, is collected in a cyclone, an apparatus it removes particles of dried material from the air by centrifugal force. There are approximately 90 per cent sugars, 8 per cent water, and o 2 per cent hydrochloric acid in the solid hydrolyzate.

The sugars of the hydrolyzate, which are usually glucose, fructose, nnose, galactose, and xylose (depending on the nature of the wood lrolyzed), are principally in a tetrameric form and are not directly nentable. However, by diluting the solid hydrolyzate with 3 parts volume of water and by heating the resulting solution at 120°C. for hr., the tetrameric form is converted to a fermentable form. Galac- and xylose are not fermentable by yeast.

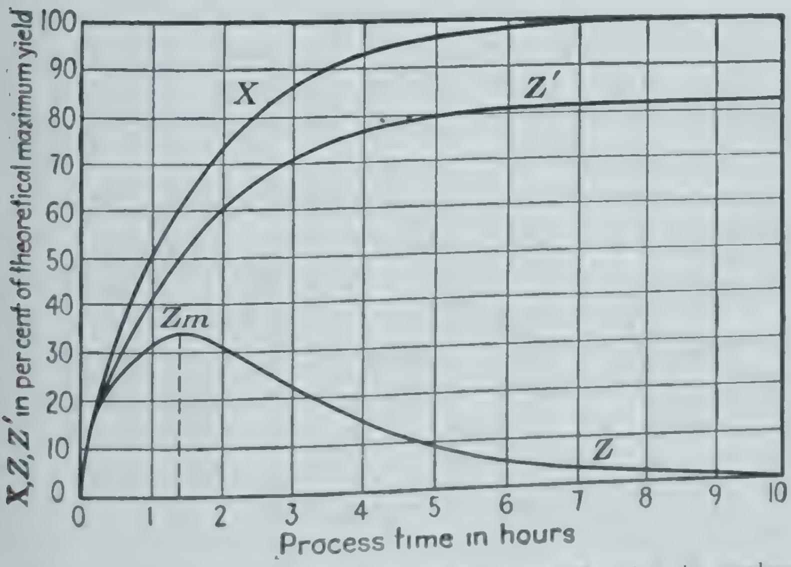
ccording to Bergius, approximately 80 per cent of the raw sugar ined by hydrolysis is fermented to alcohol, a long ton of dry wood ding 85 to 90 gal. of ethyl alcohol (190 proof). The other 20 per of the sugar may be recovered and used for fodder or for other oses.

he lignin, washed free of acid, may be used as fuel, or pure charcoal be prepared from it.

cetic acid, in quantities that correspond to those recovered during destructive distillation of wood, is obtained as a by-product of this ess.

**The Scholler-Tornesch Process.**[1] In contrast to the Bergius proc- the Scholler process employs dilute acid, elevated temperatures, and n under pressure. The wood used does not have to be dried or of any ite particle size. Furthermore, no attempt is made to recover cid.

1926 and 1927 Scholler carried out research in the laboratory that



6. Graphical representation of course of saccharification in enclosed autoclave olator. [*Courtesy of H. Scholler, Chem. Ztg.*, 60: 293 (1936).]

---

Scholler, *Chem. Ztg.*, 60: 293 (1936).

indicated the value of the periodic removal of the sugar formed sure percolation. In Fig. 146 the curve $X$ represents theoretical fication; the curve $Z_1$, the course of saccharification by the pe method; and curve $Z$, the course of saccharification by a meth corresponds to the Stettin war process.

In the former industrial process, comminuted wood is carri system of conveyors to the top of a battery of percolators into is packed. Dilute acid solution is permitted to percolate interm under pressure through the heated wood, the accumulating sugar being removed periodically to prevent it from becoming destroye sugar solution is neutralized with $CaCO_3$, permitted to settle in and filtered. It may then be fermented by yeast.

As stated above, the particle size of the wood is not of great tance. Sawdust, plane dust, rasp dust, and similar types of w variable moisture content may be used directly.

Each percolator is 14 m. in length and 2.4 m. in diameter capacity for 50 cu. m. of material. It is filled loosely with the cel containing substance. After the percolator is closed, steam press applied suddenly from above upon the loosely packed material, wi result that it is compressed evenly. The percolator is filled agai the material again compressed. The procedure is repeated unt percolator is almost completely filled with the compressed cel material.

The dilute acid solution used for hydrolysis is prepared by pu sulfuric acid into water, which has previously been warmed by pa through a heat-exchange apparatus, to give an acid concentrati approximately 0.4 per cent.

This dilute acid flows through the cellulose-containing material temperature of about 170°C. and a pressure of 8 atmospheres. The solution thus formed is removed shortly, passing through the hea changer, where it gives off heat to water being warmed for the dilute solution. Hydrolytic action continues in the percolator after the re of the sugar solution, owing to the moisture, acid, heat (160 to 190 and pressure, but no additional sugar is removed until the next pa of the hydrolyzing solution.

After the sugars have been recovered from the wood waste by i mittent percolation with dilute acid, the lignin residue, which is i form of a hard cake, must be removed from the percolator. Since has a high water content and is at a temperature of about 180°C. a end of the process, the sudden release of a section in the lower porti the percolator causes an explosive expansion of the water in the l and the cake is shattered.

From the sugars obtained by the preceding method, industrially im tant alcohols, acids, or yeast may be prepared.

Ethyl alcohol yields amounting to 60 to 70 per cent of the theor are obtained by this process.

Lignin, acetic acid, furfural, and waste sugars are by-products.

**The Giordani-Leone Process.** This process, described and illus-d by Giordani (1939), makes use of 60°Bé sulfuric acid. Space does permit a detailed account of this process, but some data concerning the Bergius, and the Scholler processes are presented in Table 238.

238. PRODUCTS OBTAINED BY DIFFERENT PROCESSES FROM 100 KILOGRAMS OF DRIED WOOD *

| | Bergius, kg. | Scholler, kg. | Giordani-Leone, kg. |
|---|---|---|---|
| acid | 4 | † | 0.2 |
| al | .. | † | 2 |
| | 33 | 30 | 30 |
| sugars | 66 | 43.3 | 60 |
| fermented by yeast | 56 | 35.2 | 48 |
| not fermentable by yeast ‡ | 10 | 8.1 | 12 |
| of ethanol (liters of 100 per cent) | 35 | 22 | 30 |

epared from data presented by M. Giordani, *Chimica e industria (Milan)*, 5–272 (1939).

ta not available.

proximate figures. May be utilized by bacteria in production of butanol, etc.

sugar derived from wood by the Giordani-Leone process, that part is unfermentable by yeast may be used by *Clostridium aceto-cum* for the production of butanol, acetone, and ethanol. The effi-of the Giordani-Leone process is said to be high.

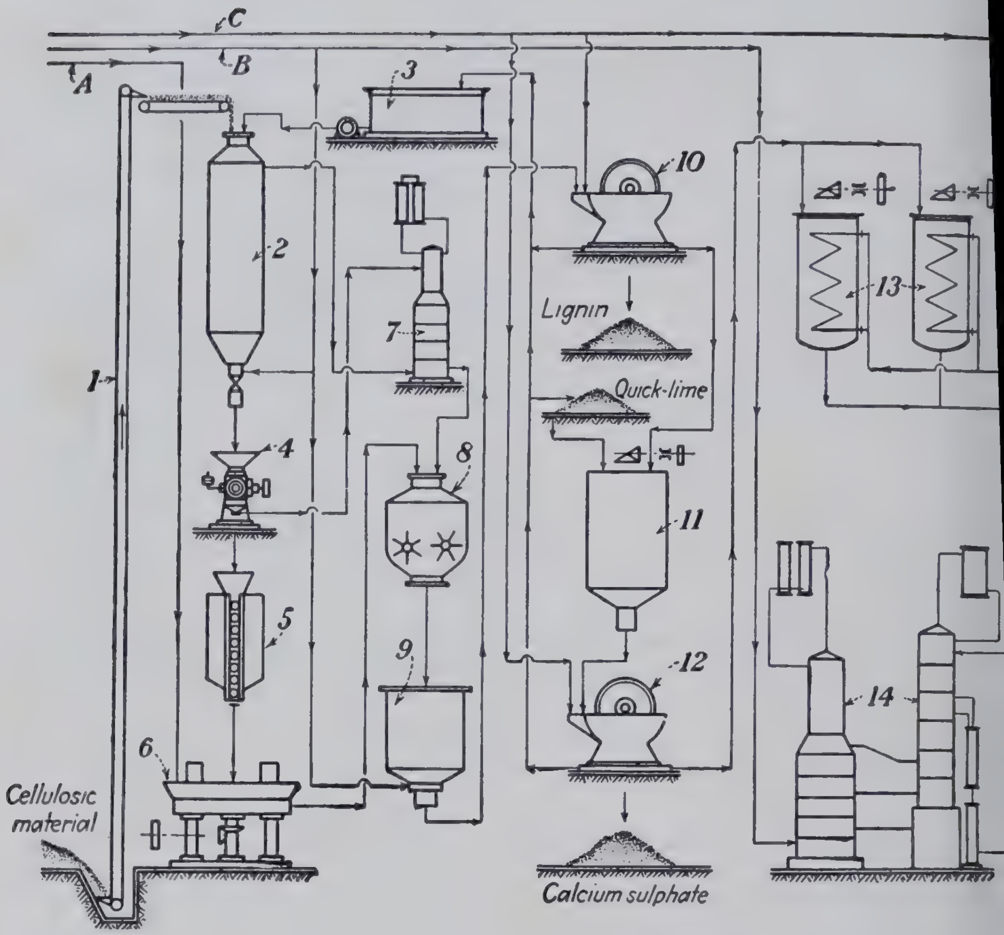
ıre 147 shows a scheme of the Giordani-Leone process for the rification of cellulosic materials.

**he Madison Wood-sugar Process.**[1] This process was developed Forest Products Laboratory of the U.S. Department of Agriculture lison, Wis. Wood waste, consisting of edgings, chipped slabs, shav-ınd sawdust, is hydrolyzed by permitting to flow continuously h it dilute sulfuric acid at temperatures of 150 to 185°C. Furfural ethanol are separated from the hydrolyzate by flashing. The yzate is then automatically neutralized with lime and filtered while pressure. The solution of wood sugars may be used for purposes s the production of ethyl alcohol by fermentation with *Saccharo-cerevisiae.*

pilot-plant equipment for hydrolyzing wood wastes is shown in 48 and 149. It consists essentially of the following elements: a hog used to break up pieces of wood waste to the desired size; facili-lifting the waste to the storage chamber which is located directly the digester; a digester constructed of silicon bronze having a y of 27 cu. ft. and of 23-in. inside diameter, with a reducing cone op and another plus a screen at the bottom; a water pump with a

---

Harris and E. Beglinger, *Report No. R-1617, Forest Products Laboratory,* Wis., June, 1946; also *Ind. Eng. Chem.*, 38(9): 890 (1946).



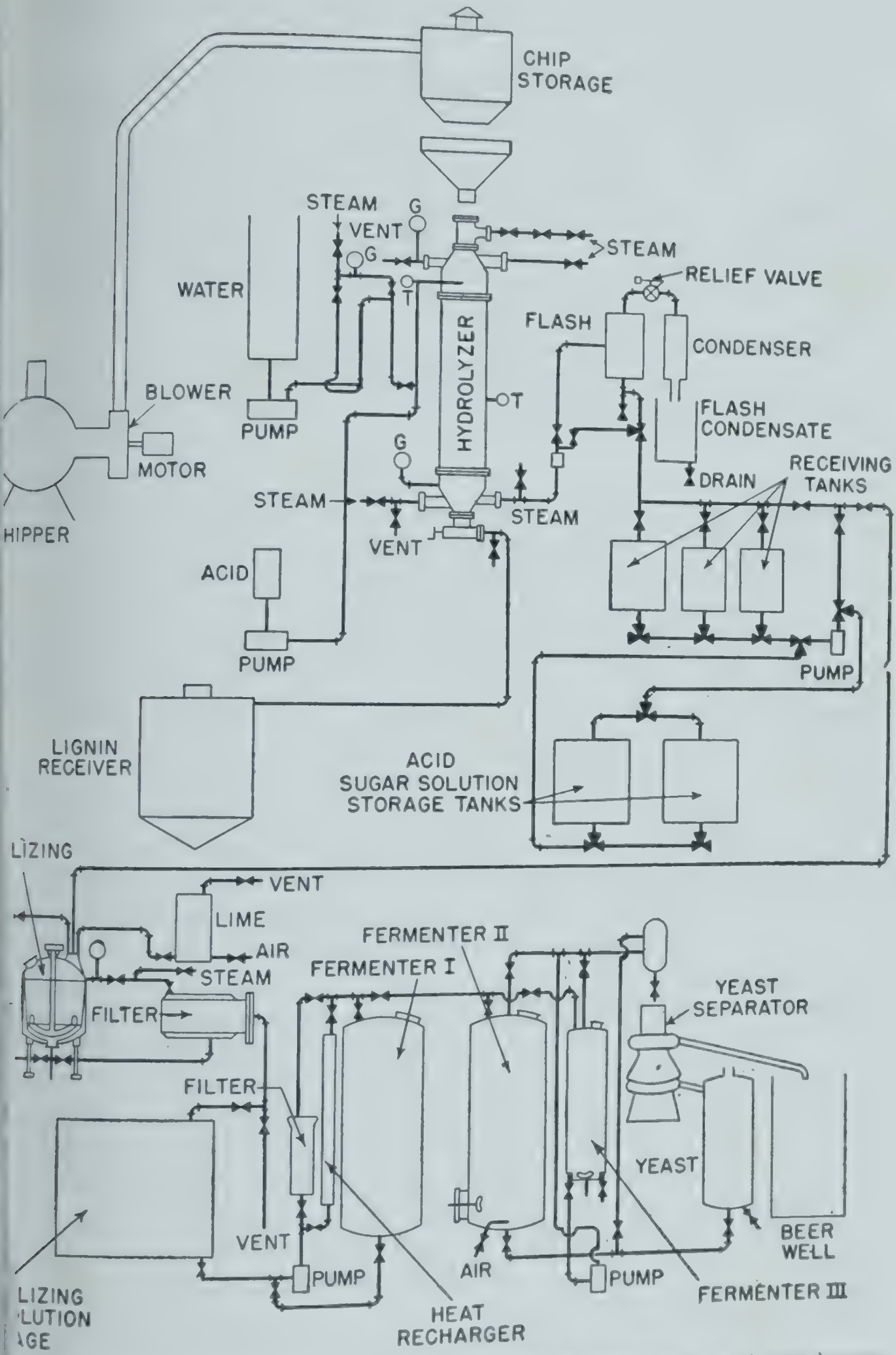
1. Elevator
2. Autoclave
3. $H_2SO_4$ (0.5 per cent) vat
4. Press
5. Drier
6. Mash
7. Furfural column
8. Pulping apparatus
9. Hydrolysis vat
10. Lignin filter
11. Neutralization vat
12. Calcium sulfate filter
13. Fermentation tank
14. Distillation column

*A*. Pipe system of $H_2SO_4$ at 60°Bé
*B*. Steam-pipe system
*C*. Water-pipe system

Fig. 147. Scheme of Giordani-Leone process for the saccharification of
materials. [*Courtesy of M. Giordani, Chimica e industria* (*Milan*), **21**: 265

capacity variable from 2 to 10 gal. per min.; an acid pump with a
variable from 0.2 to 4 gal. per min.; a switch for controlling the two
a water heater with facilities for introducing steam into the wate
located in the water line for adding acid; two inlets at the top
digester for introducing the dilute acid; a flash tank; receiving tan
mixers; a neutralizing kettle; storage tanks; and a receiver for the

The wood waste used, Douglas fir, produces best results when
in the following percentages: 25 to 30 per cent shavings, 25 to 30
sawdust, and 40 to 50 per cent edgings and slabs. These are ho
such a manner that the pieces vary from 0.25 to 1 in. in length.
wood waste containing 30 to 50 per cent of moisture is more satis
than dry wood because of the greater ease with which the acid
through the material. Bark usually constitutes 35 to 50 per cent
waste used. Yields of ethyl alcohol, used as an index of the effici


CHIP
STORAGE
STEAM
G
VENT
G
STEAM
RELIEF VALVE
WATER
T
FLASH
CONDENSER
HYDROLYZER
BLOWER
PUMP
T
FLASH
CONDENSATE
MOTOR
G
RECEIVING
TANKS
DRAIN
STEAM
STEAM
HIPPER
VENT
ACID
PUMP
PUMP
LIGNIN
RECEIVER
ACID
SUGAR SOLUTION
STORAGE TANKS
LIZING
VENT
LIME
AIR
FERMENTER II
STEAM
FERMENTER I
YEAST
SEPARATOR
FILTER
FILTER
YEAST
VENT
BEER
WELL
AIR
PUMP
PUMP
FERMENTER III
LIZING
LUTION
GE
HEAT
RECHARGER

Equipment for saccharification of wood and fermentation of wood-sugar
*(Courtesy of E. E. Harris and E. Beglinger, U.S. Dept. Agr., Forest Prod-*
*Mimeo. Report R-1617, June, 1946.)*

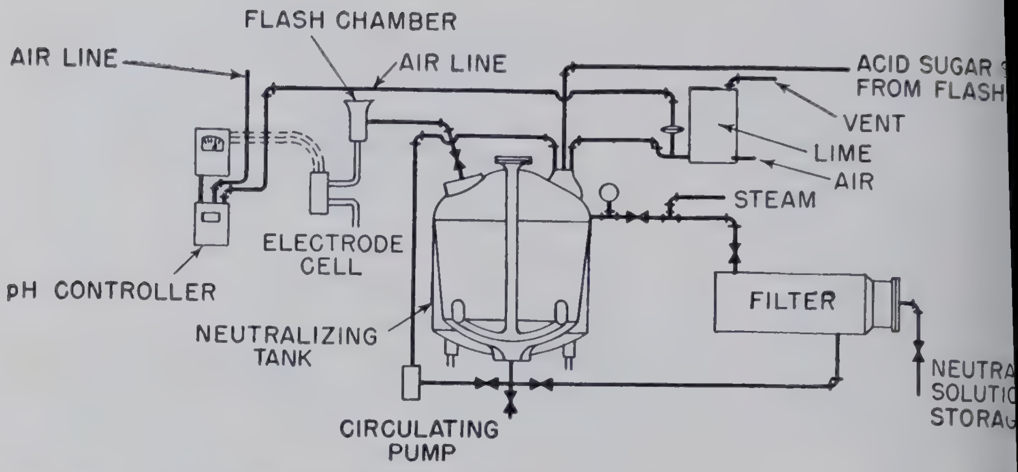


FIG. 149. Equipment for neutralization of wood sugars. (*Courtesy of E. E*
*and E. Beglinger, U.S. Dept. Agr., Forest Products Lab., Mimeo. Report*
*June,* 1946.)

hydrolysis, are calculated on the basis of dry, bark-free wood. Fro
2.5 tons of green, bark-containing wood waste is generally necess
yield a ton of bark-free wood on the dry basis.

During hydrolysis, best results are obtained when the digester is p
evenly with a uniform mixture of the hogged shavings, sawdust, ed
and slabs. The digester is filled with the wood waste, the cover is
place, and steam (at a pressure of 150 lb. per sq. in.) is introduced
idly at the top (with vents open at the bottom of the digester) unt
gauge at the top of the digester records a pressure of 10 lb. The
is then turned off. When the gauge pressure has dropped to zero, the
is opened and a new charge introduced, followed again by steam pac
After the packing is completed, steam is introduced through the bo
with the top vents open, to remove more air and to continue the heati
the wood waste. Finally, the vents are closed and steam introduc
yield a pressure of 50 lb. in the digester.

Acid is introduced into the digester as follows: At the beginning, 2
of sulfuric acid at a concentration of 1.5 to 2 per cent and at a temp
ture of 150°C. is added rapidly at the top of the digester. This am
of acid is required to bring the average acid concentration of the mois
present in the wood waste and added by the steam (about 600 lb. in
to 0.5 to 0.6 per cent. Next, sufficient sulfuric acid (at a concentratio
0.5 to 0.6 per cent and at a temperature of 150°C.) is added at the
of 12 gal. per min. to bring the ratio of dilute acid to wood (dry b
to 3:1. This usually amounts to 400 to 600 lb. of dilute acid. Fin
30 min. after the first acid has been added, the acid pump is started.
dilute acid at a concentration of 0.5 to 0.6 per cent is added at the ra
20 lb. per min. At the same time a temperature controller is set to c
the temperature in the digester to increase by 0.5°C. per min. for 30
or until a final temperature of 185°C. is obtained. This temperatu
maintained until the end of the run.

Simultaneously with the addition of the dilute acid from the pu

acid-sugar solution is removed continuously from the bottom of the
ter at a rate of about 20 lb. per min. Thus the rate of addition of
acid to the digester and that of the removal of the hydrolyzate are
ame.

e hydrolysis is continued until the average reducing-sugar concentra-
of the total combined hydrolyzate is 5 per cent. This corresponds to
continuation of the hydrolysis after the concentration of reducing
in the hydrolyzate has dropped to 0.5 per cent or less.

e hydrolyzate is passed to a flash chamber, where the pressure is
ed to 30 lb. per sq. in., permitting the steam to carry off the metha-
nd furfural. The latter are passed through a heat exchanger and,
being cooled, heat the water that is used in making the dilute acid.
e sugar solution is automatically neutralized with lime and then
d to remove calcium sulfate while still at a pressure of 30 lb.
ally, the sugar solution is cooled to 30°C. by flashing, and the pre-
te which settles out is removed by filtration.

usual time required for hydrolysis is 2.6 to 3 hr., although occa-
lly it may be slightly shorter or longer.

on of dry, bark-free Douglas fir wood waste, when hydrolyzed by
regoing process, yields 64.5 gal. of 95 per cent ethyl alcohol.

advantages of this process as compared with the Scholler process
e shorter time required for hydrolysis; the lower steam requirement;
pid removal of the sugars from the digester and the neutralization of
id, which prevents their decomposition; the production of fewer
nces inhibitory to fermentation; the longer life of the valves and
quipment, on account of fewer changes during operation; and the
yields of sugars and of alcohol from the hydrolyzed wood waste.

ant was constructed by the Government at Springfield, Ore., during
riod 1944–1947, to produce ethanol from Douglas fir waste by a
ure based on the Madison wood-sugar process. The plant was shut
fter a number of trial runs because of operational and engineering
ties.

ies based on a modified Madison wood-sugar process were carried
the Tennessee Valley Authority with the cooperation of the Forest
ts Laboratory. A number of operational difficulties were identified
solved by Gilbert et al. (1952). Based on results obtained with
lant procedures, it was ascertained that a yield of 1,470 lb. of 50
nt molasses may be obtained from 1 ton of hardwood chips. The
is referred to the publication of Gilbert and associates for further
.

**odified Rheinau Process.** A process for producing dextrose eco-
lly from waste wood has been described by Schoenemann (anony-
954). Chipped wood is hydrolyzed with 1 per cent HCl at 265°F.,
sure, to saccharify the hemicellulose. The residual wood, which is
cellulose and lignin, is centrifuged, dried, and digested with 41 per
Cl for several hours at about 70°F. The lignin is separated and
d from the top of the digester by use of water and air under pres-
may be used for the making of phenol-formaldehyde resins. The

HCl is removed from the hydrolyzate by a three-stage vacuum tr
The product is hydrolyzed to dextrose with dilute HCl. The liqu
solution is purified by passage through an ion-exchange system ar
orated under pressure. The dextrose is crystallized.

### References on Saccharifying Agents

Adams, M.: Amylases: Their Kinds and Properties and Factors Which
Their Activity, *Food Technol.*, **7**: 35–38 (1953).

Adams, S. L., B. Balankura, A. A. Andreasen, and W. H. Stark: Submerge
of Fungal Amylase, *Ind. Eng. Chem.*, **39**: 1615–1617 (1947).

Anderson, J. Ansel: "Enzymes and Their Role in Wheat Technology," Int
Publishers, Inc., New York, 1946.

Anonymous: Methods and Costs of Producing Alcohol from Grain by th
Amylase Process on a Commercial Scale, *U.S. Dept. Agr., Tech. B*
August, 1950.

Anonymous: List of Publications on Chemistry of Wood and Derived
*U.S. Dept. Agr., Forest Service, Forest Products Lab., Rept. R*238, D
1945.

Anonymous: Wood Challenges Corn as Dextrose Source, *Chem. Eng.*, F
1954, pp. 138–142.

Balankura, B., F. D. Stewart, R. E. Scalf, and L. A. Smith: Submerged Cu
Molds for Amylase Production, *Jour. Bact.*, **51**: 594 (1946).

Balls, A. K., and I. W. Tucker: *Fruit Products Jour.*, **23**: 15–16, 21 (1943).

Beresford, H., and L. M. Christensen: The Production of Ethyl Alcohol fr
Potatoes and Other Farm Crops, *Univ. Idaho Agr. Expt. Sta., Bull.* 241,

Bergius, F.: Conversion of Wood to Carbohydrates and Problems in the In
Use of Concentrated Hydrochloric Acid, *Ind. Eng. Chem.*, **29**: 247 (1937

Boulard, H.: Société d'exploitation des procédés, H. Boulard, 1931.

Boyer, J. W., and L. A. Underkofler: Mold Bran Aids Production of Grain
*Chem. Eng.*, **52**(12): 110–111 (1945).

Caldwell, M. L., and M. Adams: Action of Certain Alpha Amylases, *Adva*
*Carbohydrate Chem.*, **5**: 229–268 (1951).

Christensen, L. M.: Method of Promoting Mold Growth, U.S. Patent 2
1944.

Claassen, H.: Die Wirtschaftlichkeit der Herstellung von Holzzucker, *Chem*
**56**: 989 (1932).

Corman, J., and A. F. Langlykke: Action of Mold Enzymes in Starch Sacch
tion, *Cereal Chem.*, **25**(3): 190–201 (1948).

——— and H. M. Tsuchiya: *Cereal Chem.*, **28**: 280–288 (1951).

Dunning, J. W., and E. C. Lathrop: The Saccharification of Agricultural R
*Ind. Eng. Chem.*, **37**(1): 24–29 (1945).

Efron, A., and R. H. Blom: Production of Maltose Sirups from Granular
Flour, *Ind. Eng. Chem.*, **40**(3): 412–415 (1948).

Erb, N. M., and F. M. Hildebrandt: Mold as an Adjunct to Malt in Grain Fer
tion, *Ind. Eng. Chem.*, **38**(8): 792–794 (1946).

———, R. T. Wisthoff, and W. L. Jacobs: Factors Affecting the Production o
lase by *Aspergillus niger*, Strain NRRL 337. When Grown in Submerged
*Jour. Bact.*, **55**(6): 813–821 (1948).

Foth, G.: "Handbuch der Spiritusfabrikation," Paul Parey, Berlin, 1929.

Gallagher, F. H., H. R. Bilford, W. H. Stark, and P. J. Kolachov: *Ind. Eng.*
**34**: 1395 (1942).

Gates, R. L., and E. Kneen: Preparation of Amylase-active Concentrates from
Bran, *Cereal Chem.*, **25**(1): 1–19 (1948).

Gilbert, N., I. A. Hobbs, and J. D. Levine: Hydrolysis of Wood Using Dilu
furic Acid, *Ind. Eng. Chem.*, **44**: 1712–1720 (1952).

Giordani, M.: Alcool da materiali cellulosici, *Chimica e industria* (*Milan*), 21
272 (1939).

ve, O.: The Amylo Process of Fermentation, *Jour. Inst. Brewing*, **20**: 248–261 (1914).

., L. C., E. I. Fulmer, and L. A. Underkofler: Fungal Amylases as Saccharifying Agents in the Alcoholic Fermentation of Corn, *Ind. Eng. Chem.*, **35**: 814–818 (1943).

— and J. A. Jump: Microbial Amylase Preparations—Conversion Agents for Alcoholic Fermentation, *Ind. Eng. Chem.*, **37**: 521–525 (1945).

son, A. M., T. A. Bailey, R. C. Malzahn, and J. Corman: Plant Scale Evaluation of a Fungal Amylase Process for Grain Alcohol, *Jour. Agr. Food Chem.*, **3**(10): 366–872 (1955).

is, E. E.: Saccharification of Wood, *U.S. Dept. Agr., Forest Service, Forest Products Lab., Rept. R*1475, March, 1945.

— and E. Beglinger: Madison Wood-sugar Process, *Ind. Eng. Chem.*, **38**(9): 390–895 (1946).

—, ———, G. J. Hajny, and E. C. Sherrard: Hydrolysis of Wood. Treatment with Sulfuric Acid in a Stationary Digester, *Ind. Eng. Chem.*, **37**(1): 12–23 1945).

ey, L. F., and L. E. Wise: "The Chemistry of Wood," Rheinhold Publishing Corporation, New York, 1926.

k, M., and R. L. Shriner: Hydrolysis of Starch by Sulfurous Acid, *Ind. Eng. Chem.*, **36**(11): 1001–1003 (1944).

s, P. B., and H. P. Newton: *U.S. Dept. Agr., Misc. Publ.* 327, December, 1938.

vs, G. A.: Mold Enzymes, Produced by Continuous Tray Method, *Food Inds.*, 0: 688–690, 825, 826 (1948).

n, R., and D. F. Othmer: Wood Hydrolysis—A Continuous Process, *Ind. Eng. Chem.*, **34**(3): 314–322 (1942).

nan, F. W.: The Manufacture of Ethyl Alcohol from Wood Waste, *U.S. Dept. Agr., Misc. Bull.* 983, 1922.

ense, E. H., J. Corman, J. M. Van Lanen, and A. F. Langlykke: The Production of Mold Amylases in Submerged Culture, *Jour. Bact.*, **54**(2): 149–159 (1947).

V. E. Sohns, J. Corman, R. H. Blom, J. M. Van Lanen, and A. F. Langlykke: ain Alcohol Fermentations, Submerged Mold Amylase as a Saccharifying ent, *Ind. Eng. Chem.*, **41**(1): 100–103 (1949).

E. G., J. F. Saeman, and G. K. Dickerman: The Production of Wood Sugar in rmany and Its Conversion to Yeast and Alcohol, *FIAT Final Report No.* **499**, v. 14, 1945.

W. J., R. Evans, and A. D. Dickson: A Modification of the Kneen and dstedt Methods for the Determination of Alpha- and Beta- Amylases in ley Malt, *Cereal Chem.*, **21**: 533–539 (1944).

———, and ———: A Corrected Unit of Alpha-Amylase Activity, *Cereal em.*, **24**: 299–300 (1947).

ly, W. R.: Sugar from Wood, *Jour. Chem. Ind.* (*Trans.*), **45**: 267T (1926).

W. C.: The Application of *Taka-koji* in Distilleries, *Orig. Com. 8th Int. ng. Appl. Chem.*, **26**: 69–76 (1912).

W. L.: Production of Industrial Alcohol from Grain by the Amylo Process, *l. Eng. Chem.*, **25**: 87–89 (1933).

R. H., J. F. Saeman, H. D. Turner, and E. C. Sherrard: The Rotary Digester Wood Saccharification, *Ind. Eng. Chem.*, **37**(1): 36–43 (1945).

. L., and L. A. Underkofler: Amylolytic Factors of Bran Culture and Subged Culture, *Jour. Agr. Food Chem.*, **1**(1): 87–90 (1953).

, S.: Recent Developments in Amylase Chemistry, *Wallerstein Labs. Com- s.*, **13**(41): 89–114 (1950).

, M., S. Laufer, E. D. Stewart, and L. T. Saletan: Saccharification of Wheat Fungal Amylases for Alcohol Production, *Ind. Eng. Chem.*, **36**: 811–812 4).

: The Amylase of *Bacillus polymyxa*, *Arch. Biochem.*, **16**(3): 349–355 (1948).

J. F.: Kinetics of Wood Saccharification—Hydrolysis of Cellulose and omposition of Sugars in Dilute Acid at High Temperatures, *Ind. Eng. Chem.*, ): 43–52 (1945).

———, J. L. Bubl, and E. E. Harris: Quantitative Saccharification of Cellulose, *Ind. Eng. Chem., Anal. Ed.*, **17**(1): 35–37 (1945).

———, E. E. Harris, and A. A. Kline: Analysis of Wood Sugars, *Ind. E* *Anal. Ed.*, **17**(2): 95–99 (1945).

Sandstedt, R. M., E. Kneen, and M. J. Blish: A Standardized Wohlgem dure for Alpha-Amylase Activity, *Cereal Chem.*, **16**: 712–723 (1939).

Schoene, L., E. I. Fulmer, and L. A. Underkofler: Saccharification of Sta Mashes for the Alcoholic Fermentation Industry, Comparison of Ge charifying Agents, *Ind. Eng. Chem.*, **32**: 544–547 (1940).

Scholler, H.: Die Gewinnung von Zucker, Spiritus und Futterhefe au Rohstoff, *Chem. Ztg.*, **60**: 293 (1936).

———: French Patents 706678; 777824; and 799358; U.S. Patents 1,641,771; 1,990,097; 2,083,347; 2,083,348; 2,086,963; 2,088,977; 2,108,567; 2,123,211; 2,188,192; and 2,188,193.

Schwimmer, S.: The Malt Amylases, *Brewers Dig.*, **26**(3 and 4): 29–32T, 40 48T (1951).

Severson, G. M.: *Iowa State Coll. Jour. Sci.*, **11**: 215 (1937).

——— and K. J. Goering: Saccharification of Grain Mashes for the Alco mentation Industry. Plant-scale Use of Mold Amylase, *Ind. Eng. C* 980–985 (1946).

Sherrard, E. C., and F. W. Kressman: A Review of Wood Saccharification in the United States Prior to World War II, *U.S. Dept. Agr., Forest* *Forest Products Lab., Rept. R*1454, September, 1944.

Somogyi, M.: A New Reagent for the Determination of Sugars, *Jour. Bio* **160**: 61–68 (1945).

Stark, W. H., S. L. Adams, R. E. Scalf, and P. Kolachov: Laboratory Cookin ing, and Fermentation Procedures, *Ind. Eng. Chem., Anal. Ed.*, **15**: (1943).

Takamine, J.: Process for Producing Diastatic Product, U.S. Patent 1,054,3

———: Diastatic Product, U.S. Patent 1,054,626, 1913.

———: Enzymes of *Aspergillus oryzae* and the Application of Its Amy Enzyme to the Fermentation Industry, *Ind. Eng. Chem.*, **6**: 824–828 (19

———: Diastatic Product and the Process for Producing the Same, U.S. 1,148,938, 1915.

———: Process for Producing Diastatic Product, U.S. Patent 1,263,817, 1918.

Thorne, C. B., R. L. Emerson, W. J. Olson, and W. H. Peterson: Evalu Malts for Production of Alcohol from Wheat, *Ind. Eng. Chem.*, **37**: 1 (1945).

Toyama, N.: Amylolytic Action of *Aspergillus oryzae* in Submerged Cultu *Fermentation Technol.*, **28**(12): 445 (1950).

Underkofler, L. A.: Production of Diastatic Material, U.S. Patent 2,291,009,

———: Microbial Amylases. Their Application to Alcoholic Fermentation, *Dig.*, **17**(11): 29 (1942).

——— and E. I. Fulmer: *Chronica Botan.*, **7**: 420 (1943).

———, ———, and L. Schoene: Saccharification of Starchy Grain Mashes Alcoholic Fermentation Industry. Use of Mold Amylase, *Ind. Eng. Ch* 734–738 (1939).

——— and D. K. Roy: Crystallization of Fungal Alpha-Amylase and Limit ase, *Cereal Chem.*, **28**: 18–29 (1951).

———, G. M. Severson, and K. J. Goering: Saccharification of Grain Ma Alcoholic Fermentation, *Ind. Eng. Chem.*, **38**: 980–985 (1946).

———, ———, ———, and L. M. Christensen: Commercial Production and Mold Bran, *Cereal Chem.*, **24**(1): 1–22 (1947).

Unger, E. D.: Thesis, Case School of Applied Science, May, 1941.

———, H. F. Willkie, and H. C. Blankmeyer: The Development and Des Continuous Cooking and Mashing System for Cereal Grains, *Trans. A* *Chem. Engrs.*, **40**: 421–443 (1944).

Van Lanen, J. M., and R. H. Blom: "Report on Production and Utilization o Bran," Northern Regional Research Laboratory, Peoria, Ill., 1945.

— and E. H. Le Mense: The Production of Fungal Amylases in Submerged Culture and Their Use in the Production of Industrial Alcohol, *Jour. Bact.*, **51**: 595 (1946).

l. E. H., F. H. Schwaiger, H. G. Leonhardt, and J. A. Merten: "The Practical Brewer," Master Brewers Association of America, St. Louis, Mo., 1947.

ie, H. F.: Distillery Processes, *Chem. Eng.*, May, 1945.

— and P. J. Kolachov: "Food for Thought," Indiana Farm Bureau, Inc., Indianapolis, 1942.

lgemuth, J.: Über eine neue Methode zur quantitativen Bestimmung des dia-tatischen Ferments, *Biochem. Zeit.*, **9**: 1–9 (1908).

## CHAPTER 47

# SOME BIOCHEMICAL ENGINEERING ASPECT OF FERMENTATION

Space limitations permit only brief reference to the biochemic neering aspects of fermentation.

The application of biochemical engineering principles is esse economical production and for the obtainment of the highest yield products. It is also necessary for quality control.

The biochemical engineer controls the environment during the tion, recovery, and purification of the fermentation product. This the materials used in the construction of the fermentor, pipe lines, and other equipment; the design and construction of the fermen other facilities; the sterilization of media, equipment, and air; t trol of pH, temperature, air flow, air pressure, and other variab conditioning and aeration systems; the use of antifoam agents a tems; recording systems; and other facilities.

**Materials of Construction.** The proper selection of materials struction is of prime importance in the successful production of a of fermentation products. Treatises on chemical engineering, ma turers' data, and special trade journals are good sources of infor on this subject. Reviews concerning chemical engineering mate construction appear from time to time in *Industrial and Engi Chemistry*. These are well worth the careful attention of the read

**Fermentors.** Many fermentors have been designed for use i merged fermentation on a laboratory, semi-pilot-plant, and pilo scale. A list of some of these is presented in Table 239. Detailed d tions of a number of such fermentors will be found in a special p tion of the Rendiconti Istituto Superiore di Sanità, Rome, on pilo techniques relating to submerged fermentation. Details of the co tion of a 20-liter vat fermentor have been described by Dwor Lagoda, and Jackson (1954). An operating diagram of this fer is shown in Fig. 150. Some details of a fermentor used for te and research purposes are shown in Figs. 151 to 155 (Fuld and 1958).

Table 240 contains data relating to continuous fermentations.

**Aeration and Oxygen Transfer.** The adequate supply of ox the individual cell during fermentation is of large importance. Ta presents some references concerning aeration and oxygen transfer i merged fermentation.

239. Some References concerning Laboratory and Pilot-plant Fermentors of an Aerobic Type

| Scale | Reference |
|---|---|
| tory | Feustel and Humfeld: *Jour. Bact.,* **52**: 229 (1946). |
| | Humfeld: *Jour. Bact.,* **54**: 689 (1947). |
| | Saeman: *Anal. Chem.,* **19**: 913 (1947). |
| | Rivett, Johnson, and Peterson: *Ind. Eng. Chem.,* **42**: 188 (1950). |
| | Brown and Peterson: *Ind. Eng. Chem.,* **42**: 1823 (1950). |
| | Bartholomew, Karow, and Sfat: *Ind. Eng. Chem.,* **42**: 1827 (1950). |
| | Lumb and Fawcett: *Jour. Appl. Chem. (London),* **1**: S94–S98 (1951). |
| | Martin and Waters: *Ind. Eng. Chem.,* **44**: 2229 (1952). |
| | Hosler and Johnson: *Ind. Eng. Chem.,* **45**: 871 (1953). |
| | Chain et al.: *Rend. ist. super. Sanità,* **17**: 61 (1954). |
| | Lakata: *Appl. Microbiol.,* **2**: 2 (1954). |
| | Friedland, Peterson, and Sylvester: *Ind. Eng. Chem.,* **48**: 2180 (1956). |
| | Roxburgh, Spencer, and Sallans: *Can. Jour. Technol.,* **34**: 389 (1956). |
| | Lundgren and Russel: *Appl. Microbiol.,* **4**: 31 (1956). |
| nt or Semi- -plant: | |
| al. | Stefaniak, Gailey, Brown, and Johnson: *Ind. Eng. Chem.,* **38**: 666 (1947). |
| al. | Gordon et al.: *Jour. gen. Microbiol.,* **1**: 187 (1947). |
| ers | Garibaldi and Feeney: *Ind. Eng. Chem.,* **41**: 432 (1949). |
| l. | Fortune et al.: *Ind. Eng. Chem.,* **42**: 191 (1950). |
| l. | Blom et al.: *Ind. Eng. Chem.,* **44**: 435 (1952). |
| l. | Pfeifer, Vojnovich, and Heger: *Ind. Eng. Chem.,* **44**: 2975 (1952). |
| ers | Dworschack, Lagoda, and Jackson: *Appl. Microbiol.,* **2**: 190 (1954). |
| ers | Paladino, Ugolini, and Chain: *Rend. ist. super. Sanità,* **17**: 87 (1954). |
| ers | Chain, Paladino, Ugolini, and Callow: *Rend. ist. super. Sanità,* **17**: 132 (1954). |
| ers | Steel, Lentz, and Martin: *Can. Jour. Microbiol.,* **1**: 299 (1955). |
| ers | Kroll, Formanet, Covert, and Brown: *Ind. Eng. Chem.,* **48**: 2190 (1956). |
| ers | Nelson, Maxon, and Elferdink: *Ind. Eng. Chem.,* **48**: 2193 (1956). |
| l. | Fuld and Dunn: *Appl. Microbiol.,* **6**(1): 15 (1958). |

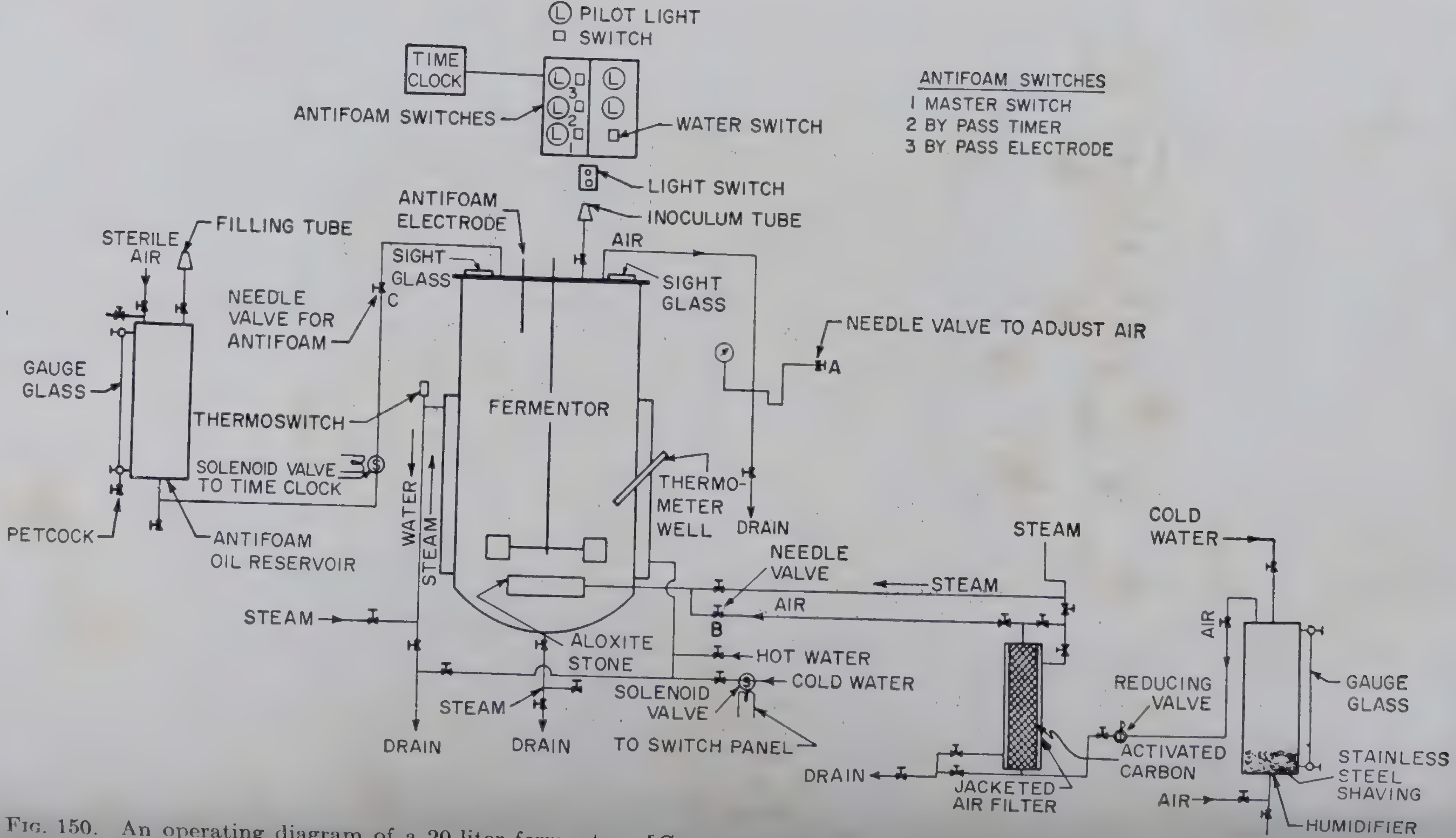
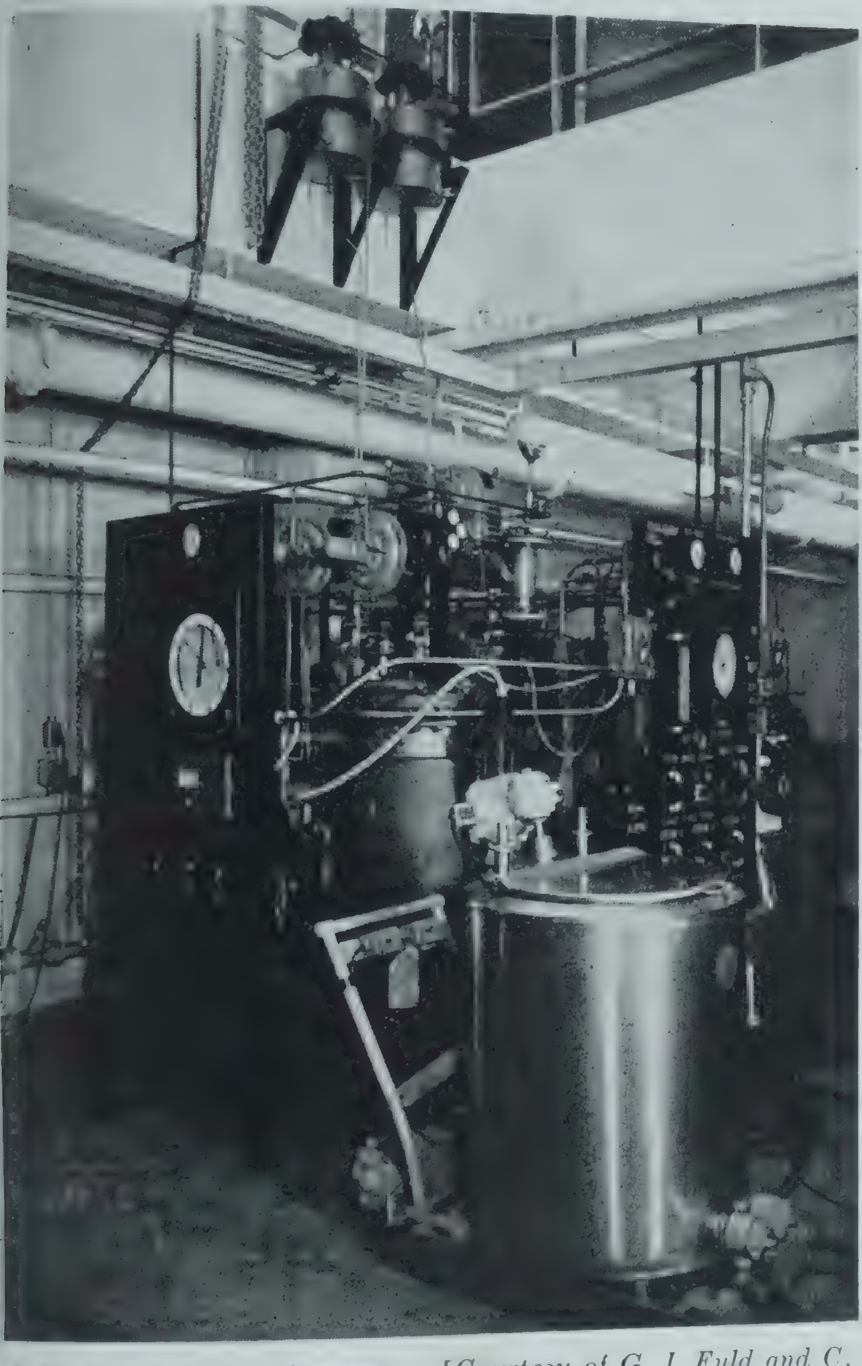

Ⓛ PILOT LIGHT
□ SWITCH
TIME CLOCK
ANTIFOAM SWITCHES
3
2
1
WATER SWITCH
ANTIFOAM SWITCHES
1 MASTER SWITCH
2 BY PASS TIMER
3 BY PASS ELECTRODE
LIGHT SWITCH
INOCULUM TUBE
ANTIFOAM ELECTRODE
FILLING TUBE
STERILE AIR
SIGHT GLASS
AIR
SIGHT GLASS
NEEDLE VALVE FOR ANTIFOAM
C
NEEDLE VALVE TO ADJUST AIR
A
GAUGE GLASS
THERMOSWITCH
FERMENTOR
SOLENOID VALVE TO TIME CLOCK
WATER
STEAM
THERMOMETER WELL
DRAIN
PETCOCK
ANTIFOAM OIL RESERVOIR
NEEDLE VALVE
STEAM
STEAM
COLD WATER
AIR
STEAM
B
ALOXITE STONE
HOT WATER
COLD WATER
AIR
STEAM
SOLENOID VALVE
REDUCING VALVE
GAUGE GLASS
DRAIN
DRAIN
TO SWITCH PANEL
ACTIVATED CARBON
DRAIN
JACKETED AIR FILTER
STAINLESS STEEL SHAVING
AIR
HUMIDIFIER

Fig. 150. An operating diagram of a 20-liter fermentor. [*Courtesy of R. G. Dworschack, A. A.*
*mentation Laboratory, Northern Utilization Research and*

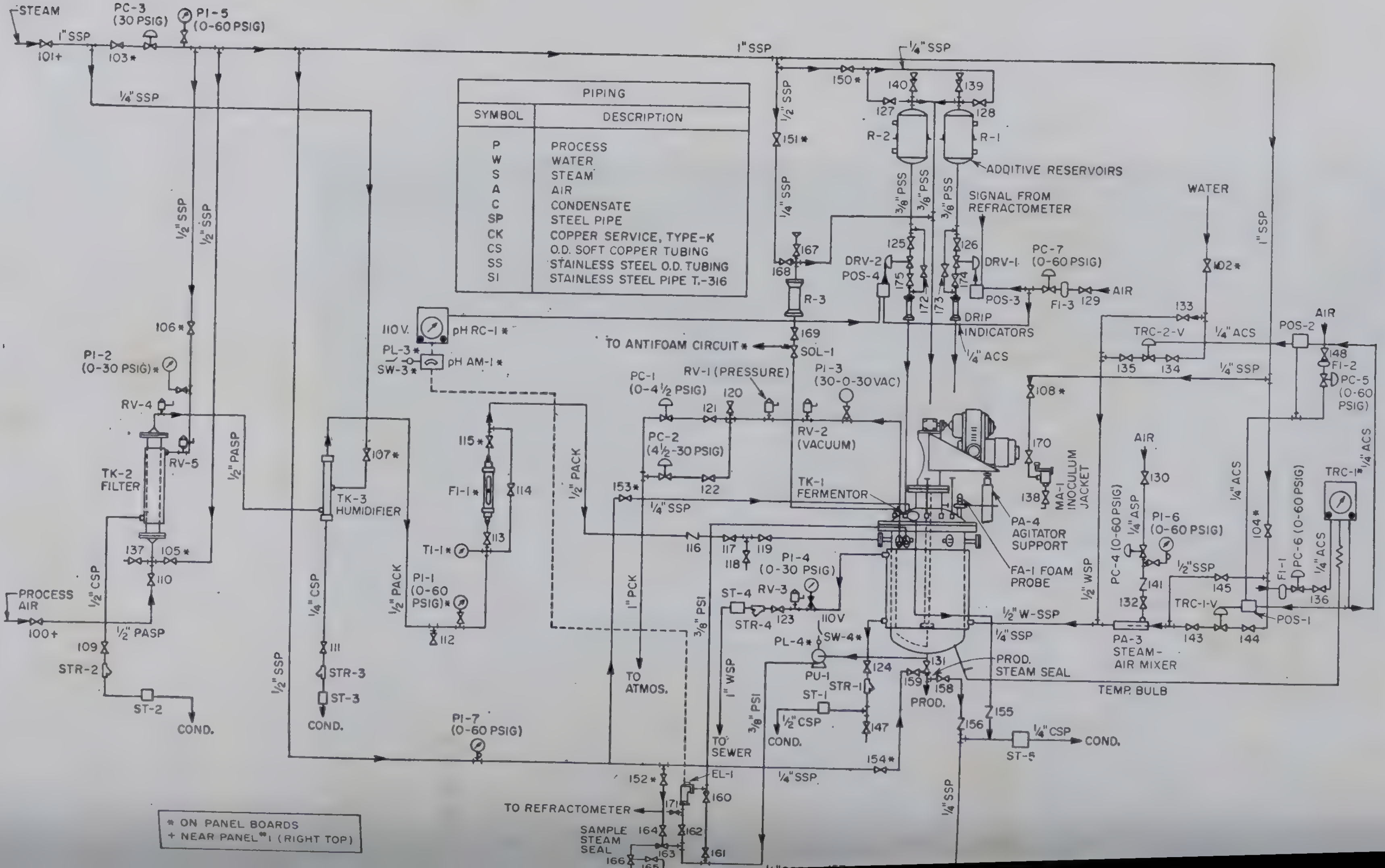


A 50-gal. pilot-plant fermentor. [*Courtesy of G. J. Fuld and C. G. Dunn,* *hnol.,* **11**: 15 (1957).]

**erilization.** In many of the modern fermentations it is essential erilized air for aeration purposes. Information relating to some used for the sterilization of air is given in Table 242.

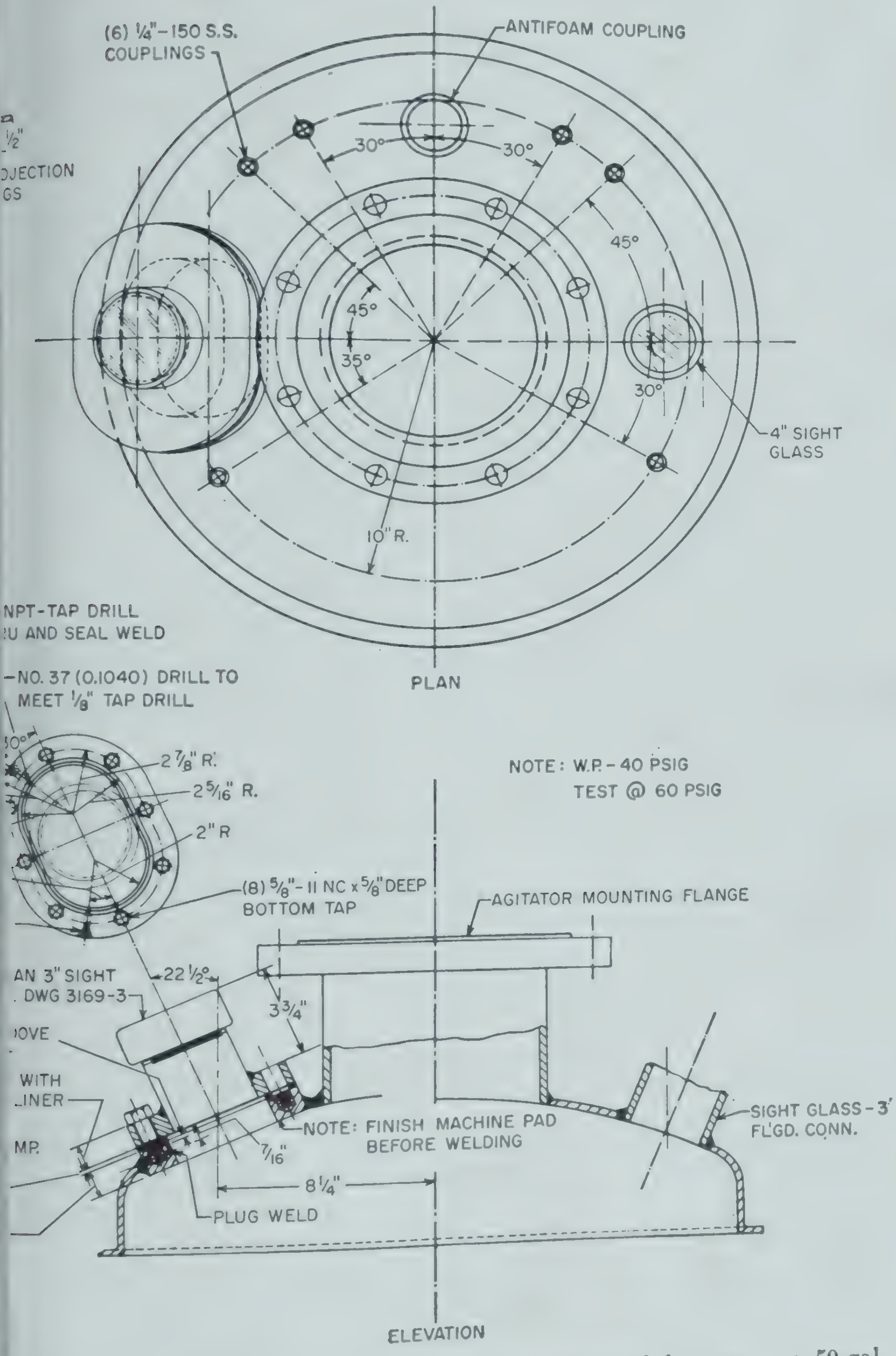
**Control.** In many of the submerged fermentations of an aerobic ontrol of foam formation is necessary. Foam may be controlled the use of chemical antifoam agents, mechanical defoaming it, and miscellaneous other methods (refer to Table 243).

**ontrol.** Although a number of fermentations are carried out he use of precise pH control, it is strongly believed that much nent in yields may be accomplished in some cases by the use of


STEAM
1" SSP
101+
PC-3 (30 PSIG)
103*
PI-5 (0-60 PSIG)
1/4" SSP
1" SSP
1/4" SSP
150*
140
139
127
128
R-2
R-1
ADDITIVE RESERVOIRS
1/2" SSP
151*
1/4" SSP
3/8" PSS
3/8" PSS
3/8" PSS
SIGNAL FROM REFRACTOMETER
WATER
1" SSP
167
168
125
126
DRV-2
DRV-1
POS-4
PC-7 (0-60 PSIG)
102*
175
172
173
174
POS-3
FI-3
129
AIR
R-3
DRIP INDICATORS
133
AIR
POS-2
169
1/4" ACS
TRC-2-V
1/4" ACS
148
FI-2
PC-5 (0-60 PSIG)
TO ANTIFOAM CIRCUIT*
SOL-1
135
134
1/4" SSP
PC-1 (0-4 1/2 PSIG)
RV-1 (PRESSURE)
PI-3 (30-0-30 VAC)
108*
121
120
RV-2 (VACUUM)
170
PC-2 (4 1/2-30 PSIG)
122
AIR
130
TRC-1*
1/4" ACS
153*
1/4" SSP
TK-1 FERMENTOR
138
MA-1 INOCULUM JACKET
PA-4 AGITATOR SUPPORT
1/4" ACS
104*
PC-4 (0-60 PSIG)
1/4" ASP
PI-6 (0-60 PSIG)
PC-6 (0-60 PSIG)
1/4" ACS
1/2" WSP
FA-1 FOAM PROBE
1/2" SSP
145
FI-1
136
116
117
119
118
PI-4 (0-30 PSIG)
141
132
TRC-1-V
POS-1
ST-4
RV-3
STR-4
123
110V
1/2" W-SSP
PA-3 STEAM-AIR MIXER
143
144
1" PCK
SW-4*
PL-4*
1/4" SSP
PROD. STEAM SEAL
TEMP. BULB
3/8" PSI
PU-1
124
131
STR-1
159
158
ST-1
PROD.
155
TO ATMOS.
1" WSP
1/2" CSP
147
156
ST-5
1/4" CSP
COND.
3/8" PSI
TO SEWER
COND.
154*
1/4" SSP
1/4" SSP
152*
EL-1
160
TO REFRACTOMETER
171
SAMPLE STEAM SEAL
164
162
163
161
166
165
110 V.
pH RC-1*
PL-3*
SW-3*
pH AM-1*
1/2" PACK
115*
FI-1*
114
113
TI-1*
PI-1 (0-60 PSIG)*
1/2" PACK
112
PI-7 (0-60 PSIG)
107*
TK-3 HUMIDIFIER
1/4" CSP
111
STR-3
ST-3
COND.
1/2" SSP
1/2" SSP
1/2" SSP
106*
PI-2 (0-30 PSIG)*
RV-4
RV-5
TK-2 FILTER
1/2" PASP
137
105*
110
PROCESS AIR
100+
1/2" CSP
1/2" PASP
109
STR-2
ST-2
COND.

| PIPING | |
|---|---|
| SYMBOL | DESCRIPTION |
| P | PROCESS |
| W | WATER |
| S | STEAM |
| A | AIR |
| C | CONDENSATE |
| SP | STEEL PIPE |
| CK | COPPER SERVICE, TYPE-K |
| CS | O.D. SOFT COPPER TUBING |
| SS | STAINLESS STEEL O.D. TUBING |
| SI | STAINLESS STEEL PIPE T.-316 |

* ON PANEL BOARDS
+ NEAR PANEL #1 (RIGHT TOP)


(6) ¼"-150 S.S. COUPLINGS
ANTIFOAM COUPLING
½"
JECTION
GS
30°
30°
45°
45°
35°
30°
4" SIGHT GLASS
10" R.
NPT-TAP DRILL
U AND SEAL WELD
NO. 37 (0.1040) DRILL TO MEET ⅛" TAP DRILL
PLAN
30°
2⅞" R.
2 5/16" R.
2" R
(8) ⅝"-11 NC x ⅝" DEEP BOTTOM TAP
NOTE: W.P. - 40 PSIG
TEST @ 60 PSIG
AGITATOR MOUNTING FLANGE
AN 3" SIGHT
. DWG 3169-3
22½°
3¾"
OVE
WITH
LINER
MP.
NOTE: FINISH MACHINE PAD BEFORE WELDING
7/16"
8¼"
PLUG WELD
SIGHT GLASS - 3' FL'GD. CONN.
ELEVATION

Drawing of a removable handhole with a steam seal for use on a 50-gal.
. [*Courtesy of G. J. Fuld and C. G. Dunn, Appl. Microbiol.,* **6**: 15 (1958).]

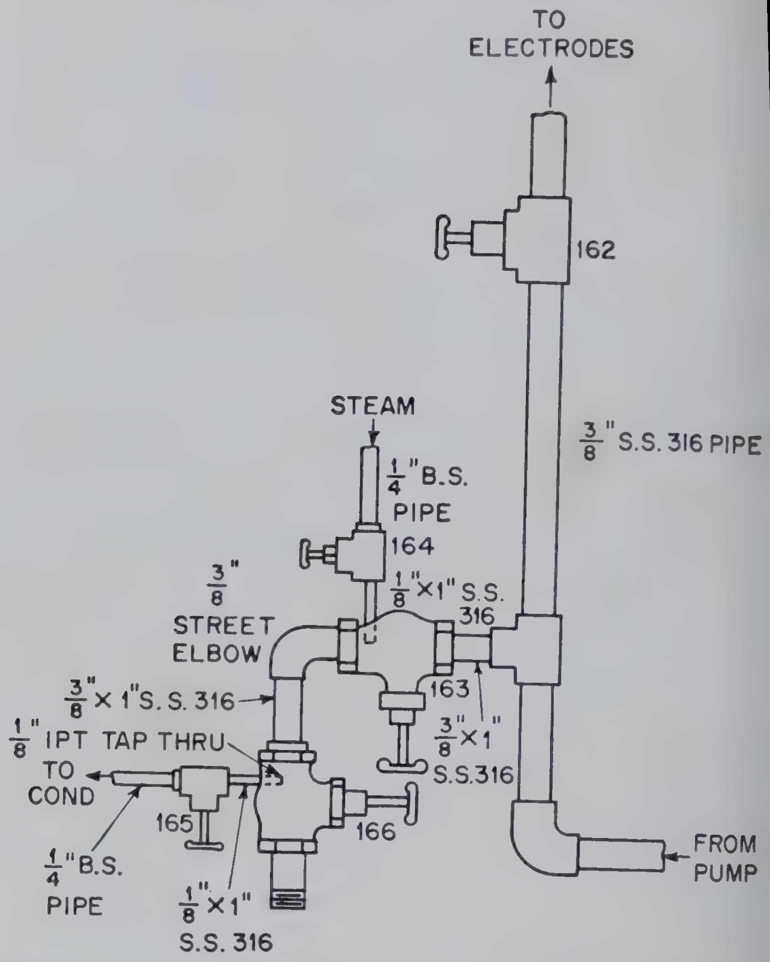

TO
ELECTRODES
162
$\frac{3}{8}$" S.S. 316 PIPE
STEAM
$\frac{1}{4}$"B.S.
PIPE
164
$\frac{3}{8}$"
STREET
ELBOW
$\frac{1}{8}$"×1" S.S.
316
163
$\frac{3}{8}$"× 1"S. S. 316
$\frac{1}{8}$" IPT TAP THRU
$\frac{3}{8}$"×1"
S.S.316
TO
COND
165
166
FROM
PUMP
$\frac{1}{4}$"B.S.
PIPE
$\frac{1}{8}$"×1"
S.S. 316

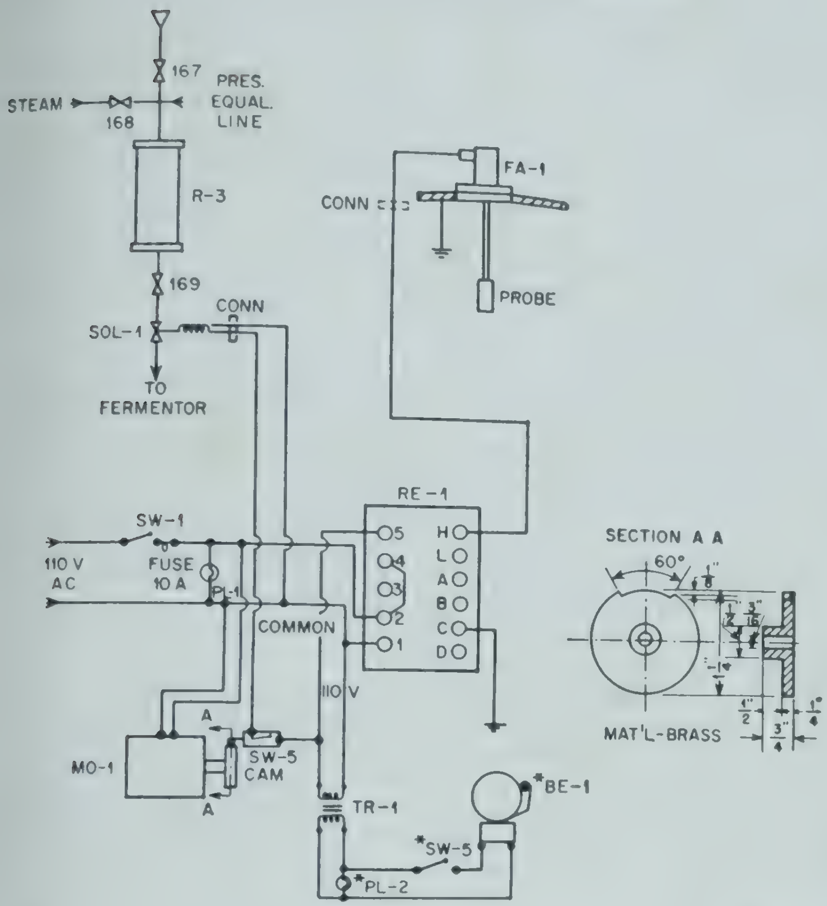
Fig. 154. Construction details of a steam-sealed sampling system for a
[*Courtesy of G. J. Fuld and C. G. Dunn, Appl. Microbiol.,* **6**: 15 (1958).]

Table 240. Some References concerning Continuous Fermentation in I
and Pilot-plant Fermentors *

| Type | References |
|---|---|
| Bacterial culture: | |
| Review of field | Novick: *Ann. Rev. Microbiol.,* **9**: 97 (19 |
| Equipment design | ——— and Szilard, *Science,* **112**: 715 (19 |
| | Monod: *Ann. inst. Pasteur,* **79**: 390 (195 |
| | Anderson: *J. Gen. Physiol.,* **36**: 733 (195 |
| Fermentation: | |
| Review | Maxon: *Appl. Microbiol.,* **3**: 110 (1955). |
| Yeast propagation | Sak: U.S. Patent 1,891,841, 1932. |
| Yeast propagation | Unger et al.: *Ind. Eng. Chem.,* **34**: 1402 |
| | Bilford et al.: *Ind. Eng. Chem.,* **34**: 1406 |
| | Keussler: German Patent 144,682, 1943. |
| Alcohol production | Altsheler et al.: *Chem. Eng. Progr.,* **43**: 4 |
| Alcohol production | Ruf et al.: *Ind. Eng. Chem.,* **40**: 1154 (19 |
| | Owen: *Sugar,* **43**: 36 (1948). |
| | Victorero: U.S. Patent 2,450,218, 1948. |

---

* G. J. Fuld and C. G. Dunn, Department of Food Technology, Mass
Institute of Technology, Cambridge, Mass., 1958.


STEAM
168
167
PRES. EQUAL. LINE
R-3
169
CONN
SOL-1
TO FERMENTOR
FA-1
CONN
PROBE
RE-1
5
4
3
2
1
H
L
A
B
C
D
SECTION A A
60°
SW-1
110 V AC
FUSE 10 A
PL-1
COMMON
110 V
A
A
MO-1
SW-5 CAM
TR-1
*BE-1
*SW-5
*PL-2
MAT'L-BRASS

5. Typical antifoam-control system for a fermentor. [*Courtesy of G. J. Fuld* *G. Dunn, Appl. Microbiol.*, **6**: 15 (1958).]

40. Some References concerning Continuous Fermentation in Laboratory and Pilot-plant Fermentors—(*Continued*)

| Type | References |
|---|---|
| | Floro et al.: *Intern. Sugar Jour.*, **50**: 234 (1948).<br>Adams and Hungate: *Ind. Eng. Chem.*, **42**: 1815 (1950). |
| essel penicillin produc- ........................ | Liebmann and De Becze: U.S. Patent 2,524,200, Jan. 11, 1949. |
| er fermentor ......... | Malmgren and Hedén: *Acta Pathol. Microbiol. Scand.*, **30**: 223 (1952).<br>Hedén, Malmgren, Sundstrom, and Tornquist: *Acta Pathol. Microbiol. Scand.*, **30**: 285 (1952). |
| ntinuous penicillin.... | Foster and McDaniel: U.S. Patent 2,458,495, 1950.<br>Maxon and Johnson: *Ind. Eng. Chem.*, **45**: 2554 (1953). |
| fermentor .......... | Elsworth and Meakin: *Chemistry & Industry*, **33**: 926 (1954).<br>Herbert, Elsworth and Telling: *Jour. gen. Microbiol.*, **14**: 601 (1956). |
| fermentor .......... | Finn and Wilson: *Jour. Agr. Food Chem.*, **2**: 66 (1954). |
| lask propagator ...... | Owen & Johnson: *Jour. Agr. Food Chem.*, **3**: 606 (1955). |

TABLE 240. SOME REFERENCES CONCERNING CONTINUOUS FERMENTATION I AND PILOT-PLANT FERMENTORS—(*Continued*)

| Type | References |
|---|---|
| Alcohol production ......... | Herman: *Przemys. Rolny i Spozyw* (1956). |
| Alcohol production ......... | Nomura: *Jour. Fermentation Assoc.* 240 (1956). |
| Alcohol production ......... | Shichiji: *Jour. Fermentation Assoc.,* 249 (1956). |
| Alcohol production ......... | Ueda: *Jour. Fermentation Assoc., Ja* (1956). |
| 2-liter fermentor ........... | Guenther and Johnson: Meeting of t Chemical Society, September, 1957. |

TABLE 241. SOME REFERENCES CONCERNING AERATION AND OXYGEN TRANS MERGED FERMENTATION *

| Subject | Reference |
|---|---|
| Aeration design in sewage treatment.... | Eckenfelder: *Chem. Eng. Pro* (1956). |
| Aeration studies on propagation of bakers' yeast ...................... | Maxon and Johnson: *Ind. E* **45**: 2554 (1953). |
| Agitation—aeration in the laboratory and in industry ........................ | Finn: *Bact. Revs.,* **18**: 254 (195 |
| Basic factors in oxygen transfer in aeration system ........................ | Ippen and Carver: *Sewage Wastes,* **26**: 813 (1954). |
| Carbon and oxidation-reduction balances | Shu and Thorn: *Can. Jour. B* 252 (1952). |
| Considerations in scale-up work ........ | Wegrich and Shurter: *Ind. En* **45**: 1153 (1953). |
| Determination of an empirical formula on the efficiency of oxygen supply of fermentor ........................ | Yamada, Takahashi, and Oka *Agr. Chem. Soc. Japan,* **27**: 7 |
| Effect of aeration on utilization of respiratory substrates................ | Rolinson and Lumb: *Jour. ge biol.,* **8**: 265 (1953). |
| Effect of air flow and agitation rates on fermentation ...................... | Bartholomew, Karow, Sfat, and *Ind. Eng. Chem.,* **42**: 1810 ( |
| Effect of physical properties on oxygen transfer in penicillin production...... | Deindoerfer and Gaden: *App biol.,* **3**: 253 (1953). |
| Efficiency of oxygen absorption in a 20-liter fermentor—studies of growth of *Aerobacter cloacae* ................... | Elsworth, Williams, and Harr *Jour. Appl. Chem.,* **7**: 261 ( 269 (1957). |

---

* C. G. Dunn and G. J. Fuld, Department of Food Technology, Mass Institute of Technology, Cambridge, Mass., 1958.

LE 241. SOME REFERENCES CONCERNING AERATION AND OXYGEN TRANSFER IN SUBMERGED FERMENTATION—(*Continued*)

| Subject | Reference |
|---|---|
| iency of oxygen in 6-liter fermentor. | Chain et al.: *Bull. World Health Organization*, **6**: 73 (1952). |
| -liquid contacting with multiple ixers ........................... | Oldshue: *Chem. Eng. Progr.*, **52**: 319 (1956). |
| facial areas in gas-liquid absorption. | Vermeulen, Williams, and Langlois: *Chem. Eng. Progr.*, **51**: 85 (1955). |
| transfer in an agitated vessel...... | Johnson and Huang: *Jour. Agr. Ind. Chem. Eng.*, **2**: 412 (1956). |
| en absorption in agitated tanks.... | Friedmann and Lightfoot: *Ind. Eng. Chem.*, **49**: 1227 (1957). |
| en absorption in shake flasks...... | Auro, Hodge, and Roth: *Ind. Eng. Chem.*, **49**: 1237 (1957).<br>Rhodes and Gaden: *Ind. Eng. Chem.*, **49**: 1233 (1957).<br>Shu: *Jour. Agr. Food Chem.*, **1**(18): 1119 (1953). |
| en absorption in deep-tank fer- ntations ........................ | Shu: *Ind. Eng. Chem.*, **48**: 2204 (1956). |
| n-absorption rate in the aerating entor without agitation.......... | Yamada, Takahashi, and Yamamoto: *Jour. Agr. Chem. Soc. Japan*, **28**: 156 (1954). |
| -absorption velocity in shaking re ........................... | Yamada, Takahashi, and Kojima: *Jour. Agr. Chem. Soc. Japan*, **26**: 427 (1952). |
| scale-up studies ................ | Karow, Bartholomew, and Sfat: *Jour. Agr. Food Chem.*, **28**(12): 95 (1956). |
| transfer ..................... | Hixson and Gaden: *Ind. Eng. Chem.*, **42**: 1792 (1950). |
| iance of agitated gas-liquid con- rs ........................... | Cooper, Fernstrom, and Miller: *Ind. Eng. Chem.*, **36**: 504 (1944). |
| graphic measurements in fer- ors .......................... | Amsz and Peppler: *Food Eng.*, **28**(12): 95 (1956). |
| versus polarographic techniques.. | Wise: *J. gen. Microbiol.*, **5**: 167 (1951). |
| ical and practical considerations fite oxidation as oxygen absorp- neasurement ................. | Schultz and Gaden: *Ind. Eng. Chem.*, **48**: 2209 (1956). |
| impellers, effect of aeration by.. | Oldshue: *Ind. Eng. Chem.*, **48**: 2194 (1956). |

Table 242. Some References concerning Air Sterilization Applicable to Lab and Pilot-plant Fermentors *

| Type | Reference |
|---|---|
| Heat of compression: | |
| Experimental studies | Stark and Pohler: *Ind. Eng. Chem.,* **42** (1950). |
| Use of | Chain et al.: *Rend. ist. super. Sanità,* (1954). |
| Ultraviolet irradiation | Mellors: *Chemistry & Industry,* **29**: 806 (19 |
| Electrostatic precipitation | Munden: *Chemistry & Industry,* **29**: 806 (19<br>———: *Jour. Appl. Chem.,* **2**: 65 (1952). |
| Slag wool filters: | |
| Experimental studies | Tejesen and Cherry: *Trans. Inst. Chem.* *London,* **25**: 89 (1947). |
| Design of experimental equipment | Cherry, McCann, and Parker: *Jour. Appl. C* **1**: S103 (1951). |
| Cellulose-type filters | Northrup: *Chem. Eng. Progr.,* **49**: 513 (1953) |
| Porcelain candles (1.5-$\mu$ pores). | Gordon et al.: *Jour. gen. Microbiol.,* **1**: 187 (1 |
| Carbon (10 × 20 mesh) filters: | |
| Experimental studies | Freedman, C. T.: Thesis, Massachusetts Insti of Technology, Cambridge, Mass., 1956. |
| Use of | Blom et al.: *Ind. Eng. Chem.,* **44**: 435 (1952).<br>Dworschack, Lagoda, and Jackson: *Appl. Mi biol.,* **2**: 190 (1954). |
| Cotton, packed filters: | |
| Use of | Brown and Peterson: *Ind. Eng. Chem.,* **42**: 1 (1950).<br>Bartholomew, Karow, and Sfat: *Ind. Eng. Che* **42**: 1827 (1950). |
| Fibrous filters (glass): | |
| Experimental | Humphrey and Gaden: *Ind. Eng. Chem.,* **47**: (1955). |
| Design procedure | Gaden and Humphrey: *Ind. Eng. Chem.,* **48**: 2 (1956). |
| Pilot-plant evaluation | Maxon and Gaden: *Ind. Eng. Chem.,* **48**: 2 (1956). |
| Phage removal—experimental | Sadoff and Almlof: *Ind. Eng. Chem.,* **48**: 2 (1956). |
| Experimental equipment—studies of various filter materials | McDaniel and Long: *Appl. Microbiol.,* **2**: (1954). |
| Sampling of air for bacteria | Bourdillion, Lidwell, and Thomas: *Jour. Hygie* **41**: 197 (1941).<br>Lidwell: *Chemistry & Industry,* **29**: 805 (1950).<br>Kluyver and Visser: *Antonie van Leeuwenho Jour. Microbiol. Serol.,* **16**: 299 (1950).<br>Goetz: *Am. Jour. Public Health,* **43**: 150 (1953)<br>Orr, Gordon, and Kordecki: *Appl. Microbiol.,* 116 (1956). |

* G. J. Fuld, Department of Food Technology, Massachusetts Institute of Tec nology, Cambridge, Mass., 1958.

LE 243. SOME REFERENCES CONCERNING FOAM CONTROL IN LABORATORY AND PILOT-PLANT FERMENTORS *

| Type | Reference |
|---|---|
| anical: | |
| ldhof-type design ........ | Saeman: *Anal. Chem.*, **19**: 913 (1947).<br>Brown and Peterson: *Ind. Eng. Chem.*, **42**: 1823 (1950). |
| rtex aeration system ..... | Paladino, Ugolini, and Chain: *Rend. ist. super. Sanità*, **17**: 87 (1954). |
| rease of pressure ........ | Gaden and Kevorkian: *Chem. Eng.*, Vol. 63, October, 1956. |
| nal: | |
| of hot wires, etc. ....... | Gaden and Kevorkian: *Chem. Eng.*, Vol. 63, October, 1956. |
| ical: | |
| eral .................... | Ross: Rensselaer Polytech. Inst. *Bull.* 63, 1950.<br>Bikerman et al.: "Foams-theory and Industrial Applications," Rheinhold Publishing Corporation, New York, 1953.<br>Ghosh and Pirt: *Rend. ist. super. Sanità*, **17**: 149 (1954).<br>Gaden and Kevorkian: *Chem. Eng.*, Vol. 63, October, 1956. |
| omatic addition of anti-am agents ............ | Everett: U.S. Patent 1,984,789, 1934.<br>Blom et al.: *Ind. Eng. Chem.*, **44**: 435 (1952).<br>Dworschack, Lagoda, and Jackson: *Appl. Microbiol.*, **3**: 190 (1954).<br>Chain et al.: *Rend. ist. super. Sanità*, **17**: 61 (1954).<br>Bartholomew and Kozlow: *Ind. Eng. Chem.*, **49**: 1221 (1957).<br>Fuld and Dunn: *Appl. Microbiol.*, **6**: 15 (1958). |

J. Fuld, Department of Food Technology, Massachusetts Institute of Tech-
Cambridge, Mass., 1958.

uously controlled pH. Some data relating to applications of auto-
pH control are presented in Table 244.

**er Types of Control Devices.** A number of other types of control
s are used in various phases of the fermentation industry, such as
rature controllers, pressure controllers, flow meters, oxygen and
dioxide analyzers, and oxidation-reduction recorders (refer to
245).

**ew Articles.** Advances in fermentation methods and techniques
biochemical engineering may be found in fermentation review
, such as those of Bacharach; Beesch and Shull (1955, 1956, 1957);
ell (1954); Dworschack (1952); Ledingham (1953); Lee (1949,
951); Perlman, Brown, and Lee (1952); Perlman, Tempel, and
(1953); and Perlman and Kroll (1954).

TABLE 244. SOME REFERENCES CONCERNING LABORATORY AND PILOT-PLANT A
OF AUTOMATIC pH CONTROL *

| Application | Reference |
|---|---|
| Bacterial culture | Longsworth and MacInnes: *Jour. Bac* (1935).<br>McKee: *Appl. Microbiol.,* **3**: 355 (1955) |
| Laboratory units—all utilize immersion-type measuring system | Brown and Peterson: *Ind. Eng. Chem.,* (1950).<br>Kempe, Halvorson, and Piret: *Ind. En* **42**: 1852 (1950).<br>Hosler and Johnson: *Ind. Eng. Chem.,* (1953).<br>Lakata: *Appl. Microbiol.,* **4**: 31 (1956).<br>Callow and Pirt: *Jour. gen. Microbiol.,* (1956).<br>Nelson, Maxon, and Elferdink: *Ind. Eng* **48**: 2193 (1956).<br>Deindoerfer and Wilker: *Ind. Eng. Ch* 1223 (1957). |
| Pilot plants—all utilize flow-type measuring system | Blom et al.: *Ind. Eng. Chem.,* **44**: 435 (19<br>Fuld and Dunn: *Appl. Microbiol.,* **6**: 15 (<br>Denison, West, Peterson, and Sylvester: of the American Chemical Society, Ne September, 1957. |
| Dynamic analysis of pH system | Fuld, Joos de ter Beerst, Gould, and Meeting of the American Chemical New York, September, 1957. |
| Review of pH control in fermentation | Kehoe: Meeting of the American Chemic ety, New York, 1957. |

---

* G. J. Fuld, Department of Food Technology, Massachusetts Institute o nology, Cambridge, Mass., 1958.

ABLE 245. SOME REFERENCES CONCERNING MISCELLANEOUS AUTOMATIC-CONTROL APPLICATIONS IN FERMENTATION *

| Subject | Measuring device utilized | Reference |
|---|---|---|
| trol of oxygen uptake—tomatic variation of agi-or speed which enables mentation to follow pre-ermined oxygen absorption ttern | Gaseous oxygen analyzer (magnetic) | |
| Shake-flask studies | | Shu: *Jour. Agr. Food Chem.*, **1**: 1119 (1953). |
| Laboratory fermentors | | ———: *Can. Jour. Technol.*, **33**: 279 (1955).<br>———: *Ind. Eng. Chem.*, **48**: 2204 (1956). |
| sis of fermentation gases ontinuous record of com-ition, no control utilized | Gaseous oxygen analyzer, carbon dioxide analyzer | Nelson, Maxon, and Elferdink: *Ind. Eng. Chem.*, **48**: 2183 (1956). |
| low into fermentor con-led by a pneumatic-type rder-controller | Automatic flow meter | Gaden: *Chem. Eng.*, Vol. 63, April, 1956. |
| tion-reduction potential ) recording only | Platinum electrode | Squires and Hosler: Meeting of the American Chemical Society, New York, September, 1957. |
| l of sugar concentration tomatic addition of sugar yeast propagation | Continuous refractive index | Fuld and Dunn: *Food Technol.*, **11**: 15 (1957).<br>——— and ———: *Ind. Eng. Chem.*, **49**: 1215 (1957). |

J. Fuld, Department of Food Technology, Massachusetts Institute of Tech-, Cambridge, Mass., 1958.

### Some References concerning Biochemical Engineering Aspects of Ferm

Bacharach, A. L.: Micro-organisms of Production, *Discovery,* **15**(7): 268–272

Beesch, S. C., and G. M. Shull: Fermentation, *Ind. Eng. Chem.,* **47**: 1857–1875 **48**: 1585–1603 (1956); **49**: 1492–1505 (1957).

Campbell, A. H.: Chemical Engineering and Antibiotic Fermentations, *Rese* 295–300 (August, 1954).

Dworschack, R. G.: The Production of Organic Acids by Fermentation, *U.S* *Agr., Bur. Agr. and Ind. Chem., Mimeographed Circ. Ser., AIC* 322, Fe 1952.

Fritts, H. W., et al.: Chemical Engineering Materials of Construction, *In* *Chem.,* **43**: 2193–2294 (1951).

Gruff, D., and K. Becher: "Bottling and Canning Beer," Siebel Publishing Co Chicago, 1956.

*Industrial and Engineering Chemistry,* Annual Reviews of Unit Processe Materials of Construction. (These reviews usually appear in the Septembe of the publication.)

Kluyver, A. J., and C. B. Van Niel: "The Microbe's Contribution to Bio Harvard University Press, Cambridge, Mass., 1956.

Kretzschmar, H.: "Hefe und Alkohol," Springer-Verlag, Berlin, 1955.

Ledingham, G. A.: Industrial Fermentations, *Ann. Rev. Microbiol.,* **7**: 433–460 (

Lee, S. B.: Fermentation, *Ind. Eng. Chem.,* **41**: 1868–1879 (1949); **42**: 1672 (1950); **43**: 1948–1969 (1951).

McKee, M. T.: Bacterial Culture with Controlled pH, *Appl. Microbiol.,* **3**: 35 (1955).

Perkins, J. C.: "Principles and Methods of Sterilization," Charles C Thomas, lisher, Springfield, Ill., 1956.

Perlman, D., W. E. Brown, and S. B. Lee: Fermentation, *Ind. Eng. Chem.,* **44**: 2012 (1952).

——— and C. L. Kroll: Fermentation, *Ind. Eng. Chem.,* **46**: 1809–1826 (1954).

———, A. E. Tempel, Jr., and W. E. Brown: Fermentation, *Ind. Eng. Chem.,* 1944–1969 (1953).

Reddish, G. F. (ed.): "Antiseptics, Disinfectants, Fungicides, and Sterilizat 2d ed., Lea & Febiger, Philadelphia, 1957.

Rushton, J. H., R. D. Boutros, and C. W. Selheimer: Mixing and Agitating, "E clopedia of Chemical Technology," The Interscience Encyclopedia, Inc., York, 1952, Vol. 9, pp. 133–164.

Spector, W. S.: "Handbook of Biological Data," W. B. Saunders Company, Phila phia, 1956.

Von Loesecke, H. W.: Some Unit Processes and Unit Operations in the Indus Utilization of Agricultural Products, *Econ. Botany,* **8**(2): 118–144 (1954).

Wilhelm, R. H., et al.: Fundamentals of Chemical Engineering, *Ind. Eng. Chem.,* 877–952 (1954).

# INDEX

tt Laboratories, 801
ham, E. P., 801, 806
ldehyde, 132, 135, 138, 139, 141, 142,
09–211, 327, 328, 416, 567, 694
ldol, 276
ls, 190
te activating enzyme, 568
tor, 447–449
sheet of vinegar production by,
449
rs, 448, 449
acid, 272, 273, 295, 474, 475, 477–
79, 564–567
o of propionic acid to, 477
acid bacteria, 428
hemical activities, 431–469
sification, 428–430
itional requirements, 430, 431
ences, 469–473
cetic acid, 272, 276
*acter aceti*, 428–431, 433, 456, 468,
3
*genum*, 433, 468
*-mucosum*, 431
*dens*, 431, 433, 468
*latum*, 166, 168, 430, 468
*lroxyacetonicum*, 459
*nicum*, 430, 456, 459, 468
*ngianum*, 428, 429, 431, 468
*nogenus*, 428–431, 456, 459
*le*, 430, 468
*ans*, 428–431
*urianus*, 428, 429, 431, 433
*rydans*, 166, 168
*ns*, 428–431, 456, 459
*s*, 428
*ydans*, 428–431, 454–462, 464–468
intenance, 458
hods of production with, 454
rients, 454
lations with, 455
e products formed by, 454, 455
*ydans* A.T.C.C. No. 621, 464
*ydans* NRRL B-72, 466
*ns*, 166, 168, 430, 468
*um*, 166, 168
*ides*, 430, 431, 456, 459

*Acetobacter xylinum*, 428–431, 433, 454, 456, 459, 460, 468
*Acetomonas*, 430
Acetone, 250–278, 285, 286, 288, 289, 295–298, 416–421
Acetone-butanol fermentation, 250–284
  biochemistry, 267–272
  details, 262–266
    bacterial contaminants, 265
    culture methods, 263
    effect of $CaCO_3$ on, 265
    methods of detecting contamination, 265, 266
    microorganisms, 262
    oxygen relationship, 264
    pH, 265
    raw materials, 263, 264
      concentration, 265
    stimulating substances, 264
    vitamins, 264
  end products, 266, 267
    acetylmethylcarbinol, 266, 267
    yellow oil, 267
  history, 250
  industrial production, 251–259
    from molasses, 252–259
      fermentation conditions, 257, 258
      filling fermentor, 257
      flow diagram, 259
      organisms producing, 252, 254, 255
      preparation of inoculum, 256, 257
      raw materials, 253, 256
      sterilization, 256
      yields, 259
    from starches, 251–253
      fermentation, 252
      filling fermentor, 252
      flow diagram, 253
      inoculum preparation, 251
      mash preparation, 252
  mechanism of formation of end products, 272–279
    scheme, of Arinstein, 273
      of Kluyver and associates, 276, 277
      of Speakman, 273
  raw materials for production, 257–262
    cassava, 202

Acetone-butanol fermentation, raw materials for production, Jerusalem artichokes, 260
    waste sulfite liquors, 257, 260
    wood sugar, 260
    xylose liquors, 260
  references, 279–284
Acetone-ethanol fermentation, 295–298
  duration, 297
  effect of calcium carbonate in, 296, 297
  end products, 295
    yields, 295, 297, 298
  influence of reaction on products of (fig.), 296
  mechanism, 298
  optimum temperature for, 297
  pH for, 296
  quantities of solvents produced in, 295
  rate of formation of acetone and alcohol in (fig.), 298
    table, 297
  raw materials used, 296
  utilization, of corncobs, 297
    of oat and peanut hulls, 298
Acetylenic compounds, 691–693
  examples of some formed by microbes (table), 692
Acetylmethylcarbinol, 266, 267, 277, 278, 400, 403, 415–421, 464, 477, 509. 510
  formula, 464
  production, 464
*Achromobacter anaerobium,* 167, 168
Achromobacteraceae, 165, 168
Acid production by *Cl. acetobutylicum,* 270
Acid saccharification, 866–885
  agricultural residues, 868–871
  grains, 866, 867
  references, 882–885
  starch-containing materials, 866, 867
  wood, 871–882
Acids formed by molds, 524, 525, 684–691
Aconitase, 568–571
Aconitic acid, 524, 557, 566, 685
  formula, 685
Acrylonitrile, 598
Actidione, 812, 815
*Actinomyces candidus,* 502
Actinomycetes, 762
Actinomycin, 763, 816
Actinone, 816
Active dry yeast, 70
Adams, G. A., 399, 401, 404–406, 408–413, 416–418
Adams, M., 862
Adams, S. L., 837, 858–861, 893
Adenosinediphosphate, 141, 240, 241
Adenosinemonophosphate, 137–139, 141, 240, 241
Adenosine-5-phosphate, 241
Adenosinetriphosphate, 137–139, 141, 241
Adenylic acid, 137–139, 141
  derivatives, 241
  (*See also* Adenosinemonophosphat
*i*-Adonitol, formula, 455
Aeration, 63, 64
  and oxygen transfer, 886
    references (table), 894, 895
*Aerobacter aerogenes,* 400–404, 482, 51
  *aerogenes* A.T.C.C. No. 12, 409, 71 712
    production of diaminopimelic a decarboxylase by, 711, 712
  *aerogenes* NRRL B-199, 401–404
  *cloacae,* 88, 714
*Aerobacter* fermentations, 347, 348
*Aeromonas hydrophila,* 400, 401, 417, 4
  production of 2, 3-butanediol by, 41 418
Aerosol O.T., 559
Agaricic acid, 424, 684, 685
  formula, 685
*Agaricus blazei,* 647, 650
  *campestris,* 647, 648, 650, 652, 653
  *placomyces,* 648
  *rodmanii,* 648
Agarwal, P. N., 27, 72, 227
Agarwala, S. C., 227
Aging of wines, 183, 184, 186, 188
Agnello, E. J., 733, 738, 740, 746
Agraffe, 185
Agricultural residues, 868–871
  products from 1 ton (table), 870
  saccharification, 868–871
    cellulose hydrolysis, 869, 870
    flow sheet of process, 871
    pentosan hydrolysis, 868, 869
    uses of hydrolyzates, 870, 871
*Agrobacterium tumefaciens,* 505
*Agrocybe dura,* 692
Aida, K., 713–716
Ainsworth, G. C., 804
Air sterilization, references (table), 896
Alanine, formula, 721
Albamycin (*see* Novobiocin)
Albury, M. N., 301, 349
Albus, W. R., 474
Alcohol-containing beverages, 51
Alcohols, 51
  from molds, 524, 525
Aldolase, 237, 615
Alexander, B. H., 371
Alexander, D. F., 765, 767, 770, 772
Alfalfa extract, 454
Alfalfa press juice, 649
Alkalex, 561
Allgeier, R. J., 484

er, R. W., 436
egnane, 3β, 7β-diol-20-one, 734
f. J. W., 896
e sparger, 860
g. C. L., 504
*aria dianthi*, 815
ler, W. B., 120, 121, 892
num powder, 865
n, P. F., 598, 602, 626–628
lite, IR-100, 561
20, 712
-50, 712
ng, H., 545
an Cyanamid Co., 768
ne, M. A., 188, 190
 acid composition of some yeasts,
 acids, 42–44, 710–722
ersion to alcohols, 43, 44
tamic acid, pathways of formation, 720–721
uction, 712–720
 *Cephalosporium* species, 713
 enzymatic processes, 720, 721
 fermentation processes, one-stage, 712, 713
wo-stage, 713–720
ine, 710–712
nmercial production, 710–712
lds, 711
nces, 721, 722
asts, 82
obenzoic acid, 27, 45, 48, 227
la, 48
ium bifluoride, 862, 865
ium gluconate, 594
ium ions, 720
ium malate, 264
ium succinate, 264
ium tartrate, 241
ericins, 796
nces, 816
., Jr., 895
cohol, 133, 142, 143
, 142, 143
ase, 157, 158, 502, 844, 860, 862
ase, 157, 158, 844, 862
process, 864
ied, 864–866
glucosidase," 862
 of representative hardwoods, woods, and agricultural residues le), 868
n, A. A., 306, 321, 813
n, A. K., 695
n, J. A., 298
n, L. G., 88, 90, 91
n, R. F., 698, 699, 770, 773, 780, 892

Andreasen, A. A., 88, 858–860
$\Delta^{1,4}$-Androstadiene-3,17-dione, 725, 751
4-Androstene-3,17-dione, 725
Androstenedione, 726, 752
Angel, C. R., 620
Angeletti, A., 229
*Anguillula aceti*, 450
Anhydroglucopyranose residues, 372
Anhydroglucose unit, 371
  degradation of periodate-oxidized, 371
Animal nutrition and antibiotics, 815, 818
Animal stick liquor, 219
Anisaldehyde, 684
Anisomycin, 763, 794, 812, 815
Anthraquinone derivatives, 684
Antibiotics, 762–835
  for animal nutrition, 815, 818
  antifungal (table), 816, 817
  for control of plant diseases, 812–815
    examples of diseases controlled (table), 814
  as food preservatives, 809–813
    data on action (table), 813
      fish, 810, 811
      fresh meat, 811, 812
      poultry, 810
  definition, 762
  factors in selection, 764
  isolation of producers, 765, 766
  isolation and purification, 785, 786
  for other purposes, 818
  penicillin production, 769–785
    commercial, 784
      flow sheet, 784
    fermentation conditions, 780–782
      aeration, 781
      agitation, 781
      antifoam agents, 781, 782
      contamination prevention, 782
      pH, 780, 781
      pressure, 781
      in synthetic medium, 782, 783
        chemical changes during (fig.), 783
        optimum feed rate, 783
      temperature, 780
    general methods, 769, 770
    inoculation methods, 774–777
    molds used, 770–773
      genealogy of Wisconsin family of strains of *P. chrysogenum* (fig.), 771
      information on some (table), 772, 773
    precursors, 778–780
    raw materials, 770–780
    sporulation media, 773, 774
  of proved value or promise (table), 763

Antibiotics, problems of research, development, manufacture (table), 763, 764
  recovery flow sheet, 788
  references, 818, 819–835
  screening techniques, 766–769
  testing, assaying, certification, 786–794
    cylinder-plate method, 787, 789–793
  tests, to determine identity, 769
    sensitivity, 794–796
"Antibiotics Annual," 800
Antifoam agents, 781, 782
Antifoam-control system for fermentor (fig.), 893
Antifungal antibiotics (table), 818, 819
Antimony lactate, 326
Antimycin A, 816
Apparatus for growing mold (fig.), 817
Appleton, G. S., 229, 232, 240
Appling, J. W., 770
D-Arabitol, 242–244
D-Arabonic acid, 508, 509
L-Arabonic acid, 508, 509
Aries, R. S., 316
Ark, P. A., 814
*Armillaria mellea,* 648
Arnold, M., 698, 699
Arnstein, H. R. V., 615, 616, 778, 780
Aronovsky, S. I., 558
Arroyo, R., 197
Arsenic, 699
  compounds, 696, 697
  qualitative test for, 697
Arsenious oxide, 697
Arsine, 697
Artichokes, Jerusalem, for ethyl alcohol production, 115–117
Arzberger, C. F., 250, 252, 296
Asai, T., 109, 626, 630, 713–715, 717
Ascorbic acid, formula, 456
Ascosin, 816
Ascospores, 21, 517
Ascosporogenous yeasts, 11
Ash constituents of yeast, 28
*Ashbya gossypii,* 218–221
Ashe, L. H., 295, 297, 298
Asparaginase, 264
Asparagine, 42, 225, 237, 264, 286, 287, 490
Aspartase, 237
Aspartic acid, 42, 264
Aspergillic acid, 685
  formula, 685
*Aspergillus,* diagram, 523
*Aspergillus albus,* 610
  *alliaceus,* 858
  *amstelodami,* 736, 738, 753
  *aureus,* 720
  *awamori,* 609
  *candidus,* 609
*Aspergillus chevalieri,* 732, 753
  *clavatus,* 526, 534, 609, 688
  *effusus,* 610
  *elegans,* 644
  *fischeri,* 88, 229, 644, 657, 661,
    fat production by, 661
  *flavipes,* 526, 753
  *flavus,* 610, 611, 685, 751, 753,
  *flavus-oryzae* group, 673
  *foetidus,* 858
  *fumaricus,* 545, 626, 688
  *fumigatus,* 609, 694, 695, 798
  *fumigatus* NRRL strain 1986,
  *giganteus,* 609, 804
  *glaucus,* 609, 688
  *gymnosardae,* 609
  *itaconicus,* 566, 598, 602, 644, 68
  *japonicus,* 545
  *luchuensis,* 672
  *melleus,* 696
  *nidulans,* 88, 610, 644, 656, 731, 737, 753
    fatty acids, 656
  *niger,* 518, 522, 523, 526, 533–54 545, 550, 551, 553, 554, 556–55 567–570, 637, 638, 684, 688, 69 731, 732, 736, 738, 745, 746, 75 800, 858
  *niger* A.T.C.C. No. 1015, 543
  *niger* 2.3, 547
  *niger* 62, 536, 537, 543
  *niger* (NRC A1–233), 553
  *niger* NRRL 3, 589, 590, 593
  *niger* NRRL 330, 318, 859, 862
  *niger* NRRL 337, 858–862
    dextrinizing enzyme productio (table), 859
  *niger* NRRL 372, 553–555
  *niger* NRRL 567, 553
  *niger* strain 67, 581, 583, 588, 589,
  *niger* strain 72–4, 546
  *ochraceus,* 696, 728, 729, 736–739,
  *oryzae,* 171, 307, 518, 522, 526, 609 614, 615, 644, 668, 676–679, 695, 847, 852, 858
  *oryzae* ISC 38B, 862
  *oryzae* NRRL 1988, 676
  *oryzae* NRRL 1989, 676
  *parasiticus,* 610
  *pseudoglaucus,* 736, 739, 753
  *repens,* 736, 739, 753
  *sydowi,* 654, 655, 657, 697
    lipids, 654, 655
    sterol content, 657
  *tamarii,* 526, 610, 614, 678
  *terreus,* 695, 696
  *terreus* A.T.C.C. 10020, 601
  *terreus* NRRL 265, 599–601
  *terreus* NRRL 265.S14, 643

*gillus terreus* NRRL 1960,
9-601
*icola*, 526
*is*, 739
*s*, 691
*tii*, 534, 547–550, 688, 695, 720, 858
oduction of citric acid with, 547–550
genous yeasts, 11
chart for penicillin, 792, 793
methods for antibiotics (table),
4
etrica, 526, 527
L., 37, 497
r, W. O., 332
, J., 502
ch, R. C., 186, 187, 189
ycin (*see* Chlortetracycline)
graphic technique, 38
E. M., 305

in content of yeasts, 82
., 239, 241, 287, 288
F. J., 302, 397
ch, A. L., 897
eae, 165
*acetoethylicus*, 295–298
cription, 295, 296
ntities of acetone and alcohol produced by, 295
*cis*, 502
*me*, 255
, 740, 753, 805
var. *mycoides* A.T.C.C. 9634,
4
*lacticus*, 306, 322
*i*, 265
*porus*, 357
*ns*, 295, 498, 501
*erium*, 482, 488, 490–492, 714
tion of cobalamin-producing strains, 489
uction of cobalamin by, 490–492
*herium* NRRL B-938, 489–491
opagation (table), 491
*tericus*, 265, 358, 497, 498, 502
(var. *B. subtilis*), 714
*ns*, 358
*xa*, 399, 404–417, 498, 501, 804
emistry of fermentation by, 415,
16
iption, 404, 405
of bacteriophage on, 414, 415
od of isolation, 404, 405
, 357
*us*, 753
*butylicum beta*, 254
357, 497–502, 805

*Bacillus subtilis* A.T.C.C. 6633, 794
  *subtilis* (Ford's strain), 400, 401, 418–420
    fermentation by, 418–420
  *tetryl*, 255
  *volutans*, 265
Bacitracin, 762, 763, 794, 815
  references, 817
Backus, M. P., 770, 771, 773
Bacteria, 247–249
  important in fermentation, 248
Bacterial amylase preparations as liquefying agents, 863
Bacterial amylases, 497–503
  α-amylase, 502
  classification, 501
  evaluation of activity, dextrinizing, 502
    starch-saccharifying, 502
  production, 497–500
    from thin stillage, 499, 500
    Waldmann process, 498, 499
    Wallerstein procedure, 498
    on wheat bran, 499
  production studies, 497, 498
  qualitative and quantitative nature, 500, 501
  uses, 503
Bacteriophage, 414, 415
  effect on strains of *B. polymyxa*, 414, 415
*Bacterium curvum*, 428, 433
  *dihydroxyacetonicum*, 428
  *flavo-zonatum*, 505
  *Hoshigaki* var. *rosea*, 462
  *industrium* var. *Hoshigaki*, 462
  *α-ketoglutaricum*, 714
  *orleanense*, 428, 433, 459
  *rhamnosifermentans*, 510–512
    description of, 511
  *succinicum*, 714
  *vini-acetati*, 428
Bakers' yeast, 60–70
  active dry, 70
  development of industry, 60
  propagation, 60–70
    general considerations, 61–65, 67
      aeration, 63, 64
      building up yeast cultures, 64, 65
      concentration of sugar, 62
      function of oxygen, 64
      nutrient materials, 61
      pH, 63, 67
      strains of yeast, 61, 62
      temperature, 64
    German process, 68, 69
    Heijkenskjöld method, 69, 70

Bakers' yeast, propagation, molasses-ammonium process, 65–69
  Vienna process, 60, 61
  yields, 70
Bakonyi, S., 298
Balankura, B., 858–860
Baldwin, I. L., 27, 543
Balling degree, 177
Balls, A. K., 68, 663
Balls-Tucker process for saccharification, 844
Bamann, E., 673
Bantz, A. C., 464
Banzon, J. R., 262
Barber, H. H., 654
Barham, H. N., 610, 611
Barker, H. A., 279
Barley, 839–842
Barnett, H. L., 698
Barnett, R. O., 373
Baronowski, T., 240
Bartholomew, W. H., 887, 894–896
Basamin-Busch medium, 382
  composition, 382
Battelle Memorial Institute, 653
Battery of fermentors (fig.), 776
Bauxite, 548
Bazooka (fig.), 376, 774
Beach, W. S., 814
Beadle, G. W., 772
Beckord, L. D., 498–502
Bedford, C. L., 36
Beech, F. W., 40, 41
Beechwood shavings, 435, 436, 441, 445
Beer, 148–173
  composition, 148, 149
  defects, 163–165
    ropiness, 166
    sarcina sickness, 166, 167
    sourness, 166
    turbidity, 163, 164, 167
  flow diagram, 152
  infection, 165–167
    agents, 165
    conditions adverse to, 165
    control, 166
    summary of information, 168, 169
  raw materials, 150, 153–155
  references, 172–173
Beesch, S. C., 251–257, 788, 897
Beet molasses, 543, 544
  production of citric acid from, 543, 544
Beet-sugar molasses, 554, 557, 559
Beglinger, E., 872, 877, 879, 880
Beijerinck, M. W., 38, 250, 285, 286, 428, 431
Beinert, H., 240
Belin, P., 654
Bell, J. M., 813
Bell, T. A., 349–351, 356–358, 36
Belval, H., 387
Benedict, R. G., 484, 762, 763,
Benjamin, C. R., 698
Bennett, W. G., 229
Bennett's agar, 485
Bentley, R., 615, 616
Bentonite, 184, 437
Benzoquinone, 453
Beresford, H., 846
Bergen, W. S., 422
"Bergey's Manual," 301, 428, 42
Bergius, F., 872, 874, 875
Bergius-Rheinau process of w charification, 874, 875
  flow diagram, 874
Bergmann, W., 228
Berl saddles, 432
Bernhauer, K., 229, 232, 460, 553, 578
  theory on citric acid formation,
Bernstein, S., 741
Berridge, N. J., 813
Bertho, A., 453
Bertrand, G., 456, 459, 461
Beyer, G. F., 212
Bhandari, R. R., 301
Bickel, A., 85
Bikerman, J. J., 897
Bilford, H. R., 109, 843, 892
Bills, C. E., 229, 232, 240
Biochemical engineering aspects mentation, 886–900
Biochemistry of acetone-butanol tation, 267–272
Bionic acids, 508, 509
  production by *Pseudomonas* 508, 509
Bios requirements of yeasts, 37
Biotin, 27, 45, 48, 227, 414
  formula, 48
Bird-Young filters, 785
Birkinshaw, J. H., 229, 518, 614, 6 644, 692, 696
Bishop, A. E., 389
Bisset, K. A., 301
Bissett, H. M., 810
Biti, 171
*Biverticillata-symmetrica,* 526, 527
Bixler, G. H., 374–376
Blackstrap molasses, 257, 316, 554
  production of lactic acid from,
Blackwood, A. C., 401, 419–421, 666
*Blakeslea trispora,* 698, 699
Bleyer, B., 545, 546
Blish, M. J., 502, 860
Bloater formation in cucumber salt 356, 357

ck, S. S., 647, 650, 652, 653
m. R. H., 422, 590, 591, 887, 896–898
nstein, R., 572
deau, C., 304
d-plasma volume extenders, 370
m, B. L., 741, 746, 747, 750
cheese, 397
n. H. B., 353
beer, 167
elmann. J. B., 804, 818
l. N., 565
tge. K., 482
aardt. A. G. J., 454
rd. M. O., 381
er. C. W.. 813
in. A., 497
E., 498
v. A. E., 493
le. R., 814
char. 548
et, R., 654
acid. 588. 589
n, 588, 589
*lia recurrentis*, 798
w. A., 619–621
f, C. S., 114
ni. W., 109
*tis cinera*, 720
house scene in modern brewery
fig.), 164
m fermentation, 161, 167
d, H., 864
on whiskey, 201
illon, R. B., 896
ux, L., 578
n, J. P., 770, 778
J. W., 810, 811
J. W., 844
n, A., 696
not, H., 644
and-butter pickles, 355
, C. R., 399
R. S., 420, 484
e, W. C., 314, 315, 317
*omyces*, 34
*ellensis*. 16. 17
*ericus*, 350. 754
*tilis*, 349–351
s' yeast, 86
ssing for food. 86
v, diagram, 151
g, 148–173
ican periodicals on, 172
g of mash. 159–161
ntation, 160, 161
ing, 161–163
ing, 153–159
ss, 149, 150
nces, 172, 173

Brian, P. W., 619, 622, 669, 762, 799, 815, 817
Bricker, H. M., 388
Briggs, G. M., 482
Briggs, M., 301
Brink, N. G., 484
Broderick, A. E., 267
Brodie, J., 770
Brown, A. J., 428, 433
Brown, C. S., 770, 887, 896, 897
Brown, E. H. C., 121
Brown, E. V., 465, 466
Brown, M. B., 770
Brown, R., 800
Brown, R. W., 277, 285, 286, 290–292
Brown, W. E., 897
Browne, C. A., Jr., 654
Browne, H. H., 109–112
*Brucella bronchiseptica*, A.T.C.C. 4617, 494
Bruins, P. F., 422
Brüll, L., 567, 568
  theory on citric acid formation, 567, 568
Brunel, J., 801
Bryan, C. S., 353, 359
Bryant, A. P., 332
Bryant, G., 381
Bubl, J. L., 873
Budding of yeast cells, 62
Buelow, G. H., 546
Buettner, L. G., 301
Bukovac, M. J., 622, 623
Bulk process for sparkling wines, 185, 186
Bull, H. B., 87
Bu'Lock, J. D., 691, 692
Bünger, H., 85
Burk, D., 42
Burkholder, P. R., 222, 762
Burns, J. A., 50, 51
Burris, R. H., 572, 770
Burton, H. S., 806
Burton, L. V., 86, 151, 309, 323
"Burtonizing," 157
2,3-Butanediol, 399–427
  determination, 422
  fermentation products, 400
  fermentations, 400–420
    (*See also* 2,3-Butanediol production)
  forms, 400–401
  historical, 399, 400
  mash clarification, 421, 422
  recovery, 422
    flow diagram, 423
  references, 424–427
  uses, 422
  yields, 402, 403
2,3-Butanediol production, 401–421
  by *Aerobacter aerogenes*, 401–404

2,3-Butanediol production, by *Aerobacter aerogenes*, from acid-hydrolyzed starches, 401–403
      effect of cooking conditions on yield, 402
      inoculum preparation, 401, 402
      mash preparations, 402, 403
    from glucose, 403, 404
    from wood hydrolyzates, 403
  by *Aeromonas hydrophila*, 417, 418
    fermentation by basal medium, 417
      conditions for, 417, 418
      products, 418
      of pyruvic acid, 418
      of xylose, 418
  by *Bacillus polymyxa*, 404–417
    basis for selection of strains, 405
      filterability of mash, 405
      stability of strain, 405
    biochemistry of fermentation by, 415, 416
    description, 404, 405
    effect on, of bacteriophage, 414, 415
    effect on yields, of aerobic fermentation, 410
      of age of inoculum, 406
      of agitation of medium, 412
      of anaerobic fermentation, 411
      of cooking time and temperature, 407, 408
      of particle size of wheat, 407
      of reduced pressures, 410, 411
      of surface-volume ratio, 409, 410
      of yeast extract and wheat fractions, 413
    fermentation in glucose-inorganic salts medium, effect on, of growth factors and nitrogen sources, 412–413
    fermentation of starch by, effect on, of growth factors, 413
    forms of diol produced by, 404
    inoculum media for, 406
    isolation of, 404, 405
      media, 405
    optimum temperature for, 409
    pH control, 408, 409
    pilot-plant production unit, 415
    preparation of fermentation mash, 407, 408
    production of acetone by, 416, 417
    raw materials utilized by, 406, 407
  by *Bacillus subtilis* (Ford's strain), 418–420
    effect of conditions of fermentation on yields, 419, 420
    effect of pH on dissimilation of glucose by, 420

2,3-Butanediol production
      *subtilis* (Ford's st
      tation by, history,
    medium, 419
  by *Serratia marcescens*, 4
    anaerobic dissimilation
      (table), 421
    fermentation by, condi
      medium, 421
    yields of end products
      (table), 421
Butanol, 250–277
Butanol-isopropanol fermen
    294
  by *B. technicus*, 291
  by *Cl. butylicum*, 285–290
  by *Cl. toanum*, 287, 288
  composition of media, 286,
  effect of adding products t
  effect of sodium bicarbonat
  general considerations, 285
  history, 285
  industrial production in Fo
      288
  nitrogen sources, 286
  references, 293, 294
  of sulfite liquors, 290
  use of wood sugars, 288–290
  yields of end products, 291,
Butkewitsch, W. S., 536, 539, 5
Butlin, K. R., 453, 454, 460
Butschek, G., 85
*n*-Butyl lactate, 326
2,3-Butylene glycol, 400–427, 45
  formula, 509
  (*See also* 2,3-Butanediol)
Butyric acid, 272, 273, 295
  anaerobes, 291
    production of isopropanol
Byssochlamic acid, 645
*Byssochlamys fulva*, 644, 645

Cacaomycetin, 816
Cacodyl oxide, 697
Cahn, F. J., 544
Cahn method for citric acid p
    544
Calam, C. T., 598, 605, 606
Calcium carbonate, 265, 295–297
  effect on acetone-butanol ferm
    265
Calcium citrate, 533, 540, 543, 5
Calcium gluconate (*see* Glucon
Calcium lactate, 311–313, 317,
    323–326
  flow sheet for production, 317
  production of white, 319, 320
Calcium lactobionate, 508

ium maltobionate, 508
*ariomyces fumago,* 695, 696
ariomycin, 695, 696
mu a, 696
well, M. L., 862
ornia sherry, 186
han, J. R., 770
w, D. S., 887, 898
*uctria decora,* 748, 754
rino, B., 732, 740
ron, E. J., 813
bell, A. H., 813, 897
bell, L. L., Jr., 813
cidin, 763, 816
*da,* 34
*cans,* 796, 800
*rea,* 71, 74, 227
*ri,* 224, 225
*liermondia,* 222–225
*i,* 83, 350–352
*oides,* 227
*lytica,* 16, 17, 227
*oderma,* 184, 228
*dotropicalis,* 110, 111, 226, 236
*herrima,* 71, 88, 228
*aufii,* 71, 88, 89
*rica,* 236
*icalis,* 16–18, 71
16, 17, 71, 72, 74, 79, 80, 83–86,
88, 227, 228, 236, 720
*noides,* 228
olasses for citric acid production,
–543
zaro reaction, 133
lli, C., 239, 242
*rellus cibarius,* 648
inose, 524, 695
acid, 190
c acid, 190
ycin, 763, 794
dioxide effect on citric acid pro-
tion by *A. wentii,* 549
lase, 237
methyl cellulose, 649
acid, 524, 683–685
la, 685
acid, 524, 683–685
la, 685
W. W., 388
s, E. H., 252, 256
acid, 524, 683–685
la, 685
acid, 524, 683–685
la, 685
ne, 28, 697–699
la, 698
ence, 698
tion, by *B. trispora,* 698, 699
pecies of *Rhodotorula,* 699

Carr, J. G., 40, 41
Carson, H. L., 30
Carson, S. F., 549, 572
Carter, H. E., 85
Carver, C. E., Jr., 894
Casein whey, 308
Casida, L. E., Jr., 710–712
Cassava, 262
Castellani, A., 511
Castille, 232
Catalase, 671
Catechol tannins, 153
Catenarin, 524, 684, 693
Cathcart, W. H., 813
Cathomycin (*see* Novobiocin)
Cavillito, C. J., 229
Cell-free extracts, 129, 130
Cellulose hydrolysis, 869–871
Celmer, W. D., 800
Cephalin, 26
Cephalosporin N, 806
*Cephalosporium acremorium,* 713
  *diospyri,* 713
  *salmosynnematum,* 713, 806
*Cephalothecium roseum,* 737, 743, 744, 751, 754
  *subverticillatum,* 754
Cerogras, 413
Chaiet, L., 492, 493
Chain, E. B., 801, 887, 895–897
Challenger, F., 564, 609, 615, 696, 697
Challinor, S. W., 770
Chamber for isolating single yeast spores, 31
Champagne, 175, 185, 186
Chang, W., 227
Chappell, C. H., 399
Charanduk, K., 639
Charging of seed tank (fig.), 776
Charles, A. F., 387, 388
Charles, J. H. V., 614
Charmat process, 185
Chatterjee, N. P., 542
Cheddar cheese, 395
Cheese, 393–398
  analyses of some varieties (table), 396
  Cheddar, 395, 396
  classification, 394, 395
  definition, 393
  description, 393
  preparation, 395–397
    formation of curd, 393, 394
    ripening, 394
    treatment of curd, 394
  references, 398
  Roquefort, 397
  sources of milk for, 393
  varieties, 393

- Cheese whey, 313
  - lactic acid from, 313
- Cheldelin, V. H., 27, 80–82, 85, 86, 469
- Chemical changes, during citric acid fermentation (fig.), 552
  - in continuous-feed glucose fermentation (fig.), 783
- Chemical soy sauce, 677
- Cherry, G. B., 896
- Chiao, J. S., 26, 227, 228
- Chinese soy sauce, 675–678
  - preparation of kojies for, 676, 677
- Chistiakov, M. P., 109
- Chlamydospore, 517
- Chloramphenicol, 794, 797, 810, 811, 815, 818
  - formula, 797
  - references, 817
- Chlorine-containing compounds, 695
- Chloroform, 224, 728
- Chlorokojic acid, 613
- Chloromycetin (*see* Chloramphenicol)
- Chlortetracycline, 807, 809, 815
  - formula, 807
  - use as food preservative, 809–811
- *Choanephora cucurbitarum,* 698
- Cholestenone, 723
- Cholesterol, 733
- Christensen, C. M., 772
- Christensen, L. M., 399, 401, 845, 846, 848–852
- Chromin, 816
- Chrysophanol, 524, 684, 693
- Chrzaszcz, T., 538, 565, 566
- Church, M. B., 519, 521, 523
- Ciegler, A., 698, 699
- Citric acid, 533–577
  - historical, 533
  - Krebs cycle and, 568–572
  - mechanism theories, 563–568
  - recovery, 540
  - references, 572–577
  - yields, 539
- Citric acid derivatives, 684
- Citric acid production, 534–562
  - by submerged-culture methods, 545–562
    - data, 562
    - drawing of 36-liter fermentor (fig.), 556
    - effect on, of alcohols and inoculums, 553–555
      - of ferrocyanide, 552, 553
      - of phosphate, 554–557
    - factors affecting, 545–547
    - inoculum preparation, 557–561
      - Moyer method, 558
      - Steel, Martin, and Lentz method, 558–561

- Citric acid production, by culture methods, inoculu tion, effect on pellet deve ferrocyanide, and pH, 559
          - and spore level, 56
        - pH and spores, 561
    - Karow and Waksman me 547–550
      - carbohydrate sources, 54
      - description of *A. wentii,*
      - effect of carbon dioxide,
      - effect of oxygen, 549
      - growth medium, 548
        - inoculation, 548, 549
    - literature citations, 546
    - pilot-plant production, 550
    - replacement medium, 547
      - effect of partial neutraliza 550
    - Shu and Johnson method, 5
      - chemical changes during f tion (fig.), 552
    - Szücs method, 550, 551
  - by surface-culture methods, 53
    - from beet molasses, 543
    - Cahn method, 544
    - from cane molasses, 541–543
    - characteristics of fermentati 541
    - on industrial scale, 544, 545
    - media, 535
    - significant factors in fermen 534–539
      - inorganic salts, 535–537
      - organisms, 534–535
      - oxygen supply, 538, 539
      - pH, 537
      - rate of increase in titrable mat weight, and pH (fig.), 542
      - ratio surface area to volum 538
      - sugar, 535
      - temperature, 539
- Citrus molasses, 222, 489, 491
- Citrus press water, 650, 652
- Citrus waste press juice, 81–83
  - as source of food yeast, 81–83
- Ciusa, R., 567, 568
  - theory on citric acid, 567, 568
- Claassen, H., 872
- Claborn, H. V., 306, 323, 324
- Clark, A. B., 563
- Clark, F. E., 488
- Clark, F. M., 699
- Clark, R. K., Jr., 806
- Clark, T. F., 588, 593
- Classification, cheeses, 394
  - fungi (fig.), 516

ssification, yeasts, 33–38
*iceps purpurea*, 228
vton, C. N., 814
nent, M. T., 546, 553, 562
ıdenning, K. A., 422
ton, C. E., 142, 279, 479, 721
ical dextran, flow sheet for production (fig.), 374
*ocybe diatetra*, 692
*tridium acetobutylicum*, 250, 267–273, 277, 278, 304, 305, 498, 503, 504, 871
*etobutylicum* A.T.C.C. 4259, 504
*ıylosaccharobutyl-propylicum*, 255
*ijerinckii*, 290
*ulinum*, 812
*tylicum*, 260, 285–287, 289–292
lescription, 285, 286
*tyricum*, 482
*rifactor*, 255
*hlearium*, 483
*num*, 287, 288
*ridium* strains for neutral solvent production (table), 254, 255
ey, J. E., 384
erbuck, P. W., 539, 683, 685, 770, 01
lamin, 482, 488–492
duction by *B. megatherium*, 488–492
tamines, 609
*liascus*, 36
ıg, A. T., 211
ıg-Lilly process for glycerol, 21
oil, 221
r, R. C., 40, 41
). M., 814
yme systems, 239
ıulone, 153
l, R. D., 401–403, 599, 600, 605, 643, 5, 767, 770, 773, 774, 777, 778, 780,
W. E., 240
435
H., 387
sworth, D. R., 724, 732, 738
*ɔtrichum antirrhini*, 741, 754
*ia umbulata*, 648
*ipes*, 648
l Food Yeast, Ltd., 85
k, S. C., 673
ssed-yeast manufacture, flow diagram, 67
sing enzyme, 568–571
phore, 523
ım, 523
. F., 673
in, W., 210, 211
r, R. A., 814

Construction materials for lactic acid, 324, 325
Contamination detection, 265, 266
Continuous fermentation, 313, 314
Continuous production of fodder yeast, 83–85
Continuous sterilizer, 252
Continuous tray method for mold enzymes, 666–669
  process diagram, 667
Control tests in yeast manufacturing, 68
Converzyme, 863
Conway, H. F., 380, 382, 386
Cook, A. H., 25, 33
Cook, E., 800
Cook, R. P., 770
Cooker detail (fig.), 848
Cooling coils in yeast fermentation, 78
Cooper, C. M., 895
Copper, 537
Copper lactate, 325, 326
*Coprinus comatus*, 648
  *similis*, 694
Corbellini, A., 613
Cordon, T. C., 317, 318
Coremia, 528
Cori, C. F., 140, 141
Corman, J., 381, 388, 666, 715, 858, 859, 862
Corn, 252, 253
  for ethyl alcohol production, flow sheet of process, 113
Corn oil, 221, 223
Corn-steep liquor, 61, 219, 253, 260, 313, 379, 381, 387, 412, 430, 454, 465, 506–509, 548, 558, 602, 603, 643, 652, 659, 699, 711, 712, 727, 773, 774, 777–780, 859
Corn-steep liquor solids, 485
Corn-steep water, 286
Corn sugar, lactic acid from, 314–316
  flow diagram, 315
Corncobs, 260, 296, 297, 435, 868–871
Cornstarch, 414
Cortical hormones, 751
Corticosterone, 738, 744, 745, 746, 748
Cortisone, 724, 744, 748, 749, 751
*Corynebacterium diphtheriae*, 710, 798
  *michiganense*, 505
  *simplex* A.T.C.C. 6946, 748, 749, 754
Cosbie, A. J. C., 166
Costilow, R. N., 349–352
Cottonseed meal, 778
Coughlin, F. M., 352
Covert, A. S., 887
Cox, C. P., 301
Cox, R. S., 814
Coyne, F. P., 645
Cozymase, reduced, 140

Craig distribution system, 786
Crampton, C. A., 202, 204
Cross, B. E., 619, 620
Crotonaldehyde hydrate, 276
Crowded-plate method, 765
Cruess, W. V., 180–183, 185, 186, 193, 363–365, 434, 437, 442
Cryptococcaceae, 10, 34
Cryptococcoideae, 34
*Cryptococcus*, 34
  *neoformans*, 16, 17, 796
Cuban high-test molasses, 542
Cucumber fermentation, 344–352, 354
  changes during, 344–349
    biochemical, 345, 346
    microbiological, 346–349
Culture tray (fig.), 668
*Cunninghamella blakesleeana*, 738–740, 750, 752, 754
Curd treatment, 394
Currie, J. N., 461, 533, 535, 537, 541, 579
Currie medium for citric acid, 535
Curtis, P. J., 619, 620
*Curvularia lunata*, 733, 734, 738, 740, 741, 745, 750, 752, 754
  *pallescens*, 735, 754
Custers, M. Th. J., 36
Cycloheximide, 763
Cyclopaldic acid, 524, 686
  formula, 686
Cyclopolic acid, 524, 686
  formula, 686
Cycloserine, 797
  references, 817
Cylinder-plate method, 787–793
*Cylindrocarpon radicicola*, 754
Cynodontin, 524, 684, 693
Cystine content of yeasts, 227, 228
Cytochrome c reductase, 237
Czapek's medium, 519, 644, 765
Czapek-Dox solution agar, 599
Czoklich, C., 446

*Dactylium dendroides*, 727, 736, 737, 744, 754
*Daedalea juniperina*, 692
Daft, F. S., 482
Dale, J. K., 226
D'Alelio, G. F., 607
Daly, R. E., 319
Damm, H., 661
Dangschat, G., 566
Darken, M., 483
Das Gupta, G. C., 542, 544
Davenport, A., 307, 326
Davey, V. G., 777, 782
Davidson, O., 814
Davies, R., 239
Davis, B. D., 710
Davis, G. H. G., 301
Davis, J. G., 302
Day, W. H., 818
DC Antifoam A, 73, 490, 71
DC Antifoam AF Emulsion, 4
Deatherage, F. E., 811
*Debaryomyces*, 35
  *hansenii*, 14, 15
  *matruchoti*, 228
  *membranaefaciens* var. *Holla* 350–352
De Becze, G., 64, 893
Debittering yeast, 86, 87
Defects, in beer, 163–169
  in wine, 191–193
Degree-days, 179
Dehydrocarolic acid, 524, 684, 68
  formula, 686
1-Dehydro-compound S, 726
$\Delta^{9(11)}$-Dehydro-compound S, 750
$\Delta^{14}$-Dehydro-compound S, 750
11-Dehydrocorticosterone, 744–746
Dehydrogenation reactions of 748–750
1-Dehydro-17α-oxycorticosterone,
16-Dehydroprogesterone, 747
1-Dehydrotestololactone, 725, 726
1-Dehydrotestosterone, 752
Deibel, R. H., 301
Deindoerfer, F. H., 894
Delbrück, 304
Delwiche, E. A., 479
Demain, A. L., 39, 40, 238, 239, 35
DeMoss, J. A., 236
DeMoss, R. D., 305, 328
Deniston, G. L., 389
Denny, C. B., 813
Deoxycorticosterone, 748
11-Deoxycorticosterone, 739, 741–7
Department of Health, Education Welfare, 786, 793
*Dermocybe sanguinea*, 693
11-Desoxy-17-hydroxycorticosteron
17-Desoxysteroids, 749
Deufel, R. D., 699
Dextran building floor plan, 375
Dextrans, 370–392
  applications, 389
  bacteria-producing, 373
    medium for isolation, 373
  classification, 371
  clinical, 373
    processing, 385, 386
    specifications, 378, 379
  degrading (table), 388
  methods for partial depolymeriz 387, 388
  native, 373

trans, production, 373–386
 by enzymatic synthesis, 379–385
  flow sheet, 380
  dextransucrase for, 379
   formation (fig.), 381
  process in brief, 379
 by fermentation, 374–379
  flow sheet, 374
  process, in brief, 374
   in detail, 374–379
erences, 389–392
ucture, 370–372
ransucrase, 373, 379–385
duction, 379–385
it, 383
for synthesizing dextrans, 383, 384
rinizing activity, evaluation, 502
rolactic acid, 320
tyl, 190, 509, 510
mula, 510
sis, 130
zable enzymes, 141
inopimelic acid, 710–712
version to lysine, 712
arboxylase, 712
nula, 712
luction by *E. coli* A.T.C.C. 12,408, 711
monium acid phosphate, 61, 314, 16, 319
maceous earth, 162, 310, 377, 543
l itaconate, 598
pua, A., 536
man, G. K., 78, 79
n, A. D., 860
*ella lycopersici*, 749, 750, 754
*kii*, 742, 754
turonic acid, 238, 239
oxyacetone, 459, 460, 615
ula, 459
iction from glycerol, 459, 460
imum conditions, 459, 460
anisms for, 459
from glycerol, 460
oxyacetone phosphate, 132, 137, , 140–142
Dihydroxy-compound S, 726, 733,

vdroxyphthalic acid, 524
la, 524
Dihydroxyprogesterone, 736
Dihydroxyprogesterone, 725, 736, 739, 744
ihydroxyprogesterone, 725, 731, 736, 739
ihydroxyprogesterone, 739, 740
ihydroxyprogesterone, 731
ihydroxyprogesterone, 732
streptomycin, 794, 815

α-γ-Diketoadipic acid, 563
Dill, 354
Dill pickles, 341, 344, 354, 355
 genuine, 354
 imitation, 354
Dilution-plate method, 30
Dimethylallylarsine, 697
Dimethyl itaconate, 598
Dimethyl-*n*-propylarsine, 697
Dimethyl selenide, 695
Dimler, R. J., 384
Dinaburg, A. M., 109
2,4-Dinitrophenol, 242
Dioxane, 387
Diploid, 23
 illegitimate, 23, 24
 legitimate, 23, 24
Dirr, K., 85
Distilled spirits, production, 195
Distillers' dried solubles, 859, 860
Distillers' solubles, 485, 486, 808
Distilling industries, 195
Divelbliss, C. L., 809
Divisions of penicillia, 526, 527
Dixon, M. B., 328
Dodecanol, 73
Dodson, R. M., 730
Doelger, W. P., 533–535, 537–542
Doelger medium for citric acid, 535
Donald, G. M. S., 211, 213
Donker, H. J. L., 301, 399, 404, 405
*Donovania granulomatis*, 798
Dorough, G. L., 607
Dorrell, W. W., 536, 543, 561
Dowling, H. F., 808
Dubourg, E., 326
Duchenne, J. O., 215
Duclaux, E., 250
Dufrenoy, J., 765, 770, 778, 784, 785, 804
Dugan, R., 792
Duggar, B. M., 765, 807
Dulaney, E. L., 229–232, 240, 710, 727, 728, 732, 736–739, 741, 744, 745
Dunegan, J. C., 814
Dunn, C. G., 25, 26, 50, 71, 72, 85, 285, 546, 605, 770, 784, 816, 886, 888–894, 896–899
Dunn, M. S., 301
Dunning, J. W., 260, 868–871
Duomycin (*see* Chlortetracycline)
Durbin, C. G., 809
Dworschack, R. G., 886–888, 896, 897

"E" medium, 766
Eble, T. E., 798
Ebner, H., 445–448
Edamine, 727–728
Edestin, 839

Effect of variables on separation of pentosans from hexosans (table), 868
Effront, J., 497
Efron, A., 422
Egg whites, treatment with glucose oxidase, 669, 671
Ehrlich, F., 143, 625, 626, 630
Ehrlich, J., 797
Eichinger, E. C., 229, 657
Eijkmann, C., 630
Eisenberg, G. M., 800
Eisenman, W., 464
Eisner, H., 802
Elder, A. L., 770
Elements in yeast ashes, 47
Elferdink, T. H., 887
Elliker, P. R., 302
Ellis, C., 607
Elmer, L. S., 441
Elsworth, R., 893, 894
Elvehjem, C. A., 47
Elving, F., 545, 546
Emard, L. O., 351
Embden, G., 129, 142
Emde, H., theory of, 566, 572
Emerson, R. L., 770
Emmenthaler cheese, 474, 477
Emmerling, O., 250
Emmons, C. W., 37, 762
Emodic acid, 524, 684, 686, 693
  formula, 686
Emodin, *frangula,* 524, 684, 693
*Endamoeba histolytica,* 798, 800
*Endomyces,* 33
*Endomycetoideae,* 33
*Endomycopseae,* 35
*Endomycopsis,* 35
  *fibuliger,* 14, 15, 228
  *magnusii,* 228
  *ohmeri,* 31, 350–352
Enebo, L., 88, 90, 91
Energy value of substances, 9
Engineering flow diagram of 50-gal. pilot-plant fermentor (fig.), 890
Engle, H., 814
English, A. R., 800
Enzymatic hydrolyzate of casein, 485
Enzymatic yeast hydrolyzate, 222
Enzymes, 6, 236–239
  energy relationships, 7
  production, by bacteria, 497–503
    by molds, 666–675
    by yeasts, 236–239
  references, 245, 679–687
Eoff, J. R., 212, 213
*Epicoccum oryzae,* 747, 755
Epicorticosterone, 737
11-Epicorticosterone, 746
Epi-F, 726, 731, 737, 738
11-Epi-hydrocortisone, 743, 744
Epoxidation of steroids, 750
14α,15α-Epoxido-compound S, 750
9β,11β-Epoxido-compound S, 750
14α,15α-Epoxido-hydrocortisone, 750
*trans*-L-epoxysuccinic acid, 642, 643
  formula, 642
  production, 642
  references, 643
Eppstein, S. H., 723, 727, 729, 732, 737
Epstein, I. G., 797
*Eramascoideae,* 33
Erb, N. M., 861, 862, 864
Erdin, 524, 695
  formula, 696
*Eremascus,* 33
*Eremothecium ashbyii,* 218, 219, 221,
Ergosterol, 228–232, 234, 235
  applications, 232
  determination, 232
  production, 229–232
    by molds, 229
    by yeasts, 229–232
  references, 234, 235
  structural formula, 229
Ergosteryl palmitate, 228
Ericsson, E. O., 118, 119
*Erotium chevalieri,* 736, 755
*Erwinia amylovora,* 814
  *atroseptica,* 814
  *chrysanthemi,* 814
*Erysiphe polygoni,* 815
Erythritol, 242, 243, 455
*meso*-Erythritol formula, 464
Erythrocin (*see* Erythromycin)
Erythroglaucin, 524, 684, 693
Erythromycin, 794, 798
  references, 817, 820
L-Erythrulose, 464, 465
  formula, 464
  production, 465
*Escherichia coli,* 302, 483, 493, 494, 511
    710, 711, 714, 805
  *coli* A.T.C.C. 12,408, 711
  *freundii,* 714
Esters of yeasts, 239, 241, 242
Etchells, J. L., 344–351, 353, 355–358, 360–362
Ethanol, 295–298, 400, 403, 407–410, 415–418, 420, 421
Ethyl acetate, 201, 239, 241, 524, 695
Ethyl acetoacetate, 33
Ethyl alcohol, 102–147, 553
  definitions, 102
  effect on citric acid production, 553
  facts and theories on mechanism, 126–147
    Neuberg's scheme, 131–134

yl alcohol, formulas of important intermediate and end products, 132, 133
ferences, 124–127, 144–147
es, 124
elds, from various raw materials, 122
l alcohol production, 105–121
m corn, 112–114
flow sheet, 113
m dehydrated sweet potatoes, 114, 115
atmospheric cooking of mash, 115
accharification, 115
ields, 115
ernment supervision, 123, 124
urpose, 123
m Jerusalem artichokes, 115–117
m molasses, 105–109
oncentration of sugar in, 108
ontinuous processes, 109
istillation, 109
nal treatment, 109
ow sheet, 106
olasses in, 106
utrient substances in, 108
xygen tension in, 108
reparation of starter, 106, 107
rocess, in detail, 106–109
outline, 105
ure yeast propagator in, 107
mperature for, 108
me of fermentation in, 109
ast in, 106
elds, 109
stics on, 103–105
sulfite liquor, 117, 118
mposition of sufite liquor for, 117
nditioning of sulfite liquor for, 118
w diagram, 119
cess of, at Bellingham, Wash., 118
Melle, 118
wheat, 114
aming problem, 114
ocess, 114
covery of distillers' grain and solubles, 114
whey, 109–112
rification of whey, 111
ipment, 111, 112
v sheet, 110
ose-fermenting yeasts in, 110, 111
, 111, 112
cess in brief, 109, 110
ds, 111
wood sugars, 118, 120

Ethyl lactate, 326
Ethylene chlorhydrin, 611
Ethylene chloride, 727
Ethylene glycol formula, 455
L-Ethylene oxide $\alpha,\beta$-dicarboxylic acid, 524, 686
  formula, 686
Ethyl propionate, 201
Ethyl valerate, 201
Euler, H. von, 563
Eulicin, 763, 817
Evans, J. B., 301
Evans, R., 860
Eweson, E. W., 69
Extraction section in antibiotics plant (fig.), 787

Fabian, F. W., 341, 348, 349, 353–355, 359
Färber, E., 214, 215
Farber, L., 810
Farrell, L. N., 387, 388
Farrell, M. A., 777, 778
Fat, 87–95, 654–665
  defined, 87
  nature of, produced by molds, 654–663
    *Aspergillus nidulans,* 656, 657
      *sydowi,* 654, 655, 657
    *Penicillium aurantio-brunneum,* 655, 657
      *javanicum,* 654
      *lilacinum,* 656
  produced by yeast, 87
  references, 99–101, 663–665
  some microbes capable of producing large amounts, 88
Fat content of some molds (table), 657
Fat production, 88–95, 654–663
  by *Aspergillus fischeri,* 661
  by *Fusarium* species, 661
  by *Geotrichum* species, 662, 663
    optimum conditions for, 662, 663
    from straw and oat hulls, 663
  by *Lipomyces lipoferus,* 91
  by molds, 658–663
  by *Penicillium javanicum,* 659, 661
  by *Rhodotorula glutinis,* 88–91
  by *Trichosporon pullulans,* 91–93
    by floor process, 92
    by pan process, 92, 93
  by yeasts, 88–95
    data on, 89
Faville, L. W., 373
Fawcett, R., 887
Feagin, R. C., 607
Federoff, M. W., 626
Feeney, R. E., 490, 887

Feger, V. H., 381
Fennell, D. I., 770, 772
Ferguson, W. E., 352
Fermentation, 4, 5
  definition, 5
Fermentation balance of glucose, 275, 277, 278
  using *Cl. acetobutylicum*, 277, 278
  using *Cl. saccharobutylicum*, 275
Fermentation review articles, 897
Fermentors, 886–893
  50-gal. (fig.), 889–893
  references, 887
  20-liter (fig.), 888
Fernbach, A., 250, 637
Fernstrom, G. A., 895
Ferracone, W. J., 673
Ferric sulfate, effect on α-ketoglutaric acid production by *S. marcescens*, 716
Ferrous gluconate, 594
Ferrous phosphogluconate, 594
Feustel, I. C., 490, 887
Fevold, H. L., 85
Fifty-gal. pilot-plant fermentor (fig.), 889–892
Filachione, E. M., 318, 324, 325, 328
Film-forming brine yeasts, 351
Filtration units (fig.), 786
Finch, C. A., 723
Fingerling, G., 85
Fink, H., 71, 72, 85, 92
Fink, H. H., 662, 663
Finland, M., 796, 799
Finlay, A. C., 579, 598, 602, 626–628, 765, 807, 808
Finn, R. K., 893
Firman, M. C., 810, 811
Fischbach, H., 769, 787
Fischer, H. O. L., 142, 566
Fish meal, 716
Fish preservation, 810, 811
Fisher, C. H., 318, 324, 328
Fitz, A., 250, 474, 477
Fitzpatrick, H. M., 516
Flavicidin, 803
Flavicin, 803
*Flavobacterium acetylicum*, 483
  *acidificum*, 483
  *aqatile*, 483
  *arborescens*, 483
  *devorans*, 483, 484
  *esteroaromaticum*, 483
  *flavescens*, 483
  *proteus*, 167, 169
  *solare*, 483, 484
  *suaveolens*, 483
Fleck, L. E., 868
Fleming, A., 801
Flocculation of yeasts, 49–51
  causes, 50
  characteristics, 50
  problems, 51
Florey, H. W., 502, 762, 764, 766, 7
    785, 801
Florey, K., 723
Floro, M., 893
Foam control, references (table),
Foda, I. O., 430, 431
Folic acid, 27
Folkers, K., 484
Folpmers, T., 285, 286
*Fomes laricis*, 685
Fontaine, M., 219, 221
Food and Drug Administration, 388
    790, 791
Food and fodder yeast, 71–87
  composition, 25, 26
  definition, 71
  nutritive value, 85, 86
  production, 73–85
    from citrus waste press juice,
      82
    on continuous basis, 83–85
    flow sheet, 75
    microorganisms used, 71, 72
    from molasses, 80
    from olive residue hydrolyzate, 8
    propagation conditions, 73
    from raw cane sugar, 80
    from raw materials, 72
    from sugar juice, 80
    from sulfite liquors, 73–79
    from waste protein water, 83
    from wood sugar, 79, 80
      in Germany, 79
    from wood sugar stillage, 80
  protein content of, 73
  references, 97–99
  vitamin content, 27, 82, 85, 86
  yields, 73, 74, 79, 80, 83
"Foolish-seedling disease," 619
Foot cell, 523
Forbath, T. P., 666, 808
Formanet, S., 887
Formic acid, 272, 273, 295, 296
Formosa, production of isopropanol in
    287, 288
Foster, J. W., 229, 232, 240, 546, 548, 5
    625, 626, 635, 642, 770, 774, 777, 7
    781, 802, 893
Franzen, H., 564
Fraser, D. R., 260
Frateur, J., 428, 429
  classification of acetic acid bacteria b
    429
Fratkin, S. B., 399, 412, 413
Frazier, W. C., 73, 79, 80

r, W. H., 204, 205
E. B., 264, 265, 267–272, 287–289,
1, 292, 296–298, 307, 326, 332,
6
nan, C. T., 896
an, G. G., 211, 213
nreich, E. von, 475, 476
. N., 60, 62, 70, 497
J., 723, 724, 732, 733, 735–738, 740,
, 751, 752
in, M., 606, 608, 643
nd, W. C., 887
H., 439, 440, 447–449
rator (fig.), 440
od for vinegar making, 439–441
B. M., 799
e diphosphate formula, 137
e production from mannitol, 466,

S., 305
. F., 886, 888–894, 896–899
, E. I., 213, 214, 260, 399, 401, 414,
454, 457, 459, 460, 464, 845, 846,
866, 867
H. L., 271
acid, 524, 689
la of, 687
llin, 794, 798
nces, 820
se, 568–571
acid, 524, 557, 565, 567, 569, 571,
625–629, 630, 633
tation, 625–628
la, 625
producing (table), 626
tion, 625–628
ane-Finlay-Amann process, 627,
628
. *oryzae*, 633
aksman process, 625–627
ces, 628, 629
(*see* Fumagillin)
in, 524, 684, 694
a, 694
mylases, 858–862
ting, 860, 861
tion, 858–860
omin, 815
, 572
. B., 483
197, 202, 868–871, 876, 877,

*culmorum*, 815
695
*sici*, 88
*rme*, 619, 620
357, 748, 752, 755
124, 142, 143, 197, 198, 201, 202,

G solution, 661
Gabriel, C. L., 250
Gad, A. M., 658, 659
Gaden, E. L., Jr., 222, 894–897
Gaeuskaya, M. S., 536, 539
Gailey, F. B., 770, 887
Galactocarolose, 524, 695
Galacturonic acid, 238, 239
Gall nuts, 637
Gallagher, F. H., 842
Gallic acid, 524, 637, 638, 687
  fermentation, 637, 638
  formula, 637
  references, 638
  uses, 638
Gardner, J. F., 546
Garibaldi, J. A., 484, 488–492, 887
Garrido, J. M., 658, 659
Gas produced in butanol fermentation, 271
Gastrock, E. A., 459, 579, 580, 582–587, 593
Gayon, U., 326
Geffers, H., 663
Geib, D. S., 301
Genealogy of Wisconsin family of strains of *P. chrysogenum* (fig.), 771
Generator, vinegar (fig.), 442–445
Genetics of yeasts, 22–25
Gentisic acid, 524, 684, 687
  formula, 687
Gentisyl alcohol, 524, 696
  formula, 696
Geodin, 695
*Geotrichum candidum*, 74, 88, 228, 662, 663
    fat production by, 662, 663
  *colorans*, 694
  *wallroth*, 88, 663
Geraci, J. E., 808
Geraniol, 153
Gerhardt, P., 543, 553
Germination medium, 584, 591
Gerson, M., 335
Gfeller, P., 290
Giammaria, J. J., 607
*Gibberella baccata*, 741, 755
  *fugikuroi*, 619, 621
Gibberellic acid, 619–624
  applications, 623
  effects, 622
  formula, 619
  history, 619, 620
  methods of application to plants, 621, 622
  production methods, 620, 621
    Borrow and associates, 621
    Stodola and associates, 620, 621
      fermentation, 621

Gibberellic acid, production methods, Stodola and associates, inoculum, in aerated bottles, 620, 621
      in seed tank, 621
    mold used, 620
    yields, 621
  references, 623, 624
Gibberellin A, 619
Giberrellin $A_1$, 619
Gibberellin $A_2$, 619
Gibberellin X, 619–621
Gibberellins (*see* Gibberellic acid)
Gibbs, M., 328
Gilbert, N., 881
Gill, A., 251
Gilliland, R. B., 50
Gilsonite, 193
Ginger beer, 171
Giordani, M., 873, 877, 878
Giordani-Leone process for wood saccharification, 877
  scheme (fig.), 878
Glaxo Laboratories, Ltd., 482
Glenn, W. E., 301
*Gliocladium catenulatum,* 751, 755
Gliotoxin, formula, 696
Glucogenic enzyme system, 862
Gluconic acid, 461, 462, 505, 578–597
  fermentation of concentrated glucose solutions, 588, 589
    use of calcium carbonate and boron compounds, 589
  flow sheet for production (fig.), 581
  formula, 578
  germination medium for, 584
  historical, 578
  industrial production of, 590
  inoculum preparation, 583, 584
  media for, 587
  methods of production, 578
  neutralization of, 585
  production, 579–590
    by *Aspergillus niger* NRRL 3, 589, 590
    by *A. niger* strain 67, 581–589
    by bacteria, 461, 462
    correlation of pH with glucose utilization for (fig.), 587
    flow sheet (fig.), 581
    by *Penicillium chrysogenum,* 579, 580
    by *Pseudomonas* and other species (table), 505
    rotary drum for (fig.), 586
    rotary-drum process, 581, 582
      air pressure, 583
      composition of media, 584
      organisms for, 581
      oxygen supply, 581, 582

Gluconic acid, production, se
      ous, 585, 587, 588
    shallow pan method, 579
  references, 594–597
  sporulation medium, 584
  uses, 594
  yields (table), 593
*Gluconobacter cerinum,* 715
Glucono-δ-lactone, 594
D-Glucosamine hydrochloride, 3
Glucose oxidase, 669, 671
  nature, 669
  uses, 669, 670
Glucosyl acceptor, 384
Glucuronic acid, 525, 687
  formula, 687
Glutamic acid, 143, 144
L-Glutamic acid, 712–722
L-Glutamic dehydrogenase, 721
Glutathione, 240, 550
Glutelin, 839
Glyceraldehyde, 135, 136, 141
  monophosphate, 135, 136
3-Glyceraldehyde phosphate, 137
  formula, 137
Glycerol, 133, 141, 208–217, 418–4
    711
  formula, 209
  history, 208
  production, 209–215
    by *B. subtilis,* 418–420
    by Cocking-Lilly process, 211
    by Eoff process, 212, 213
    by Freeman and Donald proce
      212
    by Fulmer-Underkofler-Hickey
      ess, 213, 214
    from hydrolyzed starch-cont
      materials, 215
    methods, 209
    from molasses, 215
    by osmophilic yeasts, 242–244
    by Shade-Färber process, 214, 2
    by sulfite process, 209–211
    from synthetic sugar, 215
  reactions in formation, 133, 134
    215
  references, 216, 217
  uses, 215
α-Glycerophosphate, 138, 141
  formula, 138
Glycogen, 524, 695
Glycolic acid, 455, 525, 567, 568, 687
  formula, 687
Glyoxylic acid, 525, 687
  formula, 687
Godkin, W. J., 813
Goering, K. J., 845, 848–853, 855
Gold, W., 796

berg, H. S., 811
ck, H., 482
dal hormones, 751
man, R. N., 812, 814
on, J. J., 887
on, R. E., 488
line, H. E., 356
o-gas," 697
ieb, D., 762
chalk, A., 626
hall, R. Y., 806
l, B. S., 611, 614, 695
, G. A., 229
, P. T., 778, 780
s for wines, recommended varieties, 8
tive value, 179
erger, R., 250
P. P., 167, 818
W. D., 239
, white, 699
es, J. E., 321
, D. E., 240
, S. R., 818
, V. W., 813
ini, B., 613
ert, M. L., 250
s, M. J., 167
fulvin, 762, 799
all, A., 370, 387
, J. J., 814
D. C., 769, 787, 794–796
medium, 548
, M., 250
, W. E., 804
cheese, 502
M., 538, 566, 567
y of, 566, 567
3. C., 542
to bacteriological examination of ed, salted, and pickled products,
mond, A., 219, 221
shaker, 243, 559
is, I. C., 279, 328
*itra esculenta*, 653
653

. J., 804
H., 92, 662, 673
effer, T. C., Jr., 86
d, E., 872
W. J., 732
G. J., 118, 120, 872
., 796
H., 94
M. F., 301
N., 430, 431

Hall, H. H., 219, 484, 485
Halvorson, H. O., 898
Hammer, B. W., 302
Hanč, O., 723
Hancock, C. W., 252
Handhole, removable, for use on 50-gal. fermentor (fig.), 891
Hannan, M. L., 84, 118, 120
Hansen, A. E., 439, 440
Hansen, E. C., 428
Hansen, H. N., 521
*Hanseniaspora*, 36
  *valbyensis*, 14, 15
*Hansenula*, 35
  *anomala*, 71, 74, 80, 227, 228, 241, 242, 351
  *saturnus*, 228, 241
  *suaveolens*, 71, 74
  *subpelliculosa*, 14, 15, 349, 350
Hanson, A. M., 858, 860, 861
Hanson, C. T. 484
Hanson, F. R., 738, 798
Hao, L. C., 845, 846, 852–854, 857, 858, 860, 863
Harden, A., 128, 129, 399, 401
Hargrove, R. E., 484
Harned, R. L., 797
Harper, G. S., 351
Harris, E. E., 79, 80, 84, 120, 873, 877, 879, 880
Harris, L. J., 482
Harrison, W. H., 337
Hashida, W., 813
Haskins, R. H., 639–641, 710
Hauge, J. G., 469
Havighorst, C. R., 180, 181
Hawley, H. B., 812, 813
Haworth, W. N., 613
Hayashi, M., 241
Hayashi, T., 620
Hayasi, T., 622, 623
Hayek, M., 867
Hazen, E. L., 800
Healey, R. P., 238
Heatley, N. G., 801
Heavy carbon acetic acid, 277
Heavy carbon butyric acid, 278
*Hebeloma sinapizans*, 648
Hedrick, L. R., 83
Hedrick, R. M., 607
Heger, E. N., 484–488, 601–604, 887
Heggested, H. E., 814
Hehre, E. J., 379, 388
Heijkenskjöld method of yeast propagation, 69
Heinrich, H. C., 482
*Helicostylum piriforme*, 732–734, 739, 740, 750, 755
Hellbach, R., 579

Hellman, N. N., 373, 384
Helm, E., 51
Helminthosporin, 524, 684, 693
*Helminthosporium avenae,* 693
  *catenarium,* 693
  *cynodontis,* 693
  *geniculatum,* 644, 695
  *gramineum,* 693
  *tritici-vulgaris,* 693
  *velutinum,* 693
*Hemophilus influenzae,* 805
Hench, P. S., 724
Hendlin, D., 484, 770
Henley, F. R., 272
Henneberg, W., 335, 428, 433
Henrici, A. T., 37, 516
Herbert, D., 498, 893
Hermann, S., 428, 462
Herrell, W. E., 801
Herrick, H. T., 210, 579, 581, 583, 593, 626, 658–660
Herwick, R. P., 801
Herzog, R. O., 563
Hesse, A., 654, 661
Hesseltine, C. W., 698, 762
Hester, A. S., 484, 485, 488
Heterofermentative lactic acid bacteria, 301
Heusghem, C., 723
Hexadecanol, 73
Hexokinases, 237
Hexose isomerase, 237
Hexosediphosphate, 128–131, 135, 136, 140, 142
Hexosemonophosphate, 131, 134, 136
Hickey, R. J., 213, 666, 723
Hickinbottom, W. J., 272
High-speed bottle filler, 163
Higuchi, K., 778, 779
Hildebrandt, F. M., 864
Hiller, M. J., 389
Hind, H. L., 171
Hines, G. E., 374–376
Hines, G. E., Jr., 226
Hinman, J. W., 799
Hirsch, A., 813
*Histoplasma capsulatum,* 796
Hitchner, E. R., 475
Hixson, A. W., 895
Hlavac, C., 728
Hobby, G. L., 806
Hock, A., 85
Hockenhull, D. J. D., 498
Hodge, H. M., 484
Hodgkin, D. C., 482
Hoeksema, H., 799
Hoerburger, W., 92, 662
Hoette, I., 18
Hof, T., 335
Hoffert, D., 93, 94
Holden, J. T., 227
Holderby, J. M., 71, 75–77, 86
Holley, H. L., 764
Holzer, E., 244
Holzer, H., 244
Homofermentative lactic acid bacteria 301, 326
  mechanism of fermentation by, 326
Homoprotocatechuic acid, 525, 687
  formula, 687
Honcamp, F., 85
Hopkins, F. G., 240
Hopkins, R. H., 157, 158, 160
Hops, 150, 152
Hordein, 839
Horecker, B. L., 279, 328
*Hormodendrum viride,* 741, 742, 755
Horne, R. W., 18
Horner, C. K., 42
Horváth, J., 724, 733
Hosler, P., 777, 781–783, 887
Houwink, A. L., 18
Hoyer, D. P., 429, 431, 452
Hromatka, O., 445–447
Huang, H. T., 720
Huang, N. H., 800
Hucker, G. J., 373
Hudson, C. S., 498
Hughes, L. P., 73, 75–77, 86
Hughes, W. L., Jr., 695
Hull, W. Q., 186, 187, 189
Hultin, E., 388
Humfeld, H., 490, 647–649, 651, 652, 887
Humphrey, A. E., 896
Humulone, 153, 159
Hungate, R. E., 893
Hunter, J. E., Jr., 267
Hussar, A. E., 764
Hwang, K., 806
Hydrocortisone, 724, 727, 738, 744, 749, 751
Hydrogen peroxide, 671
Hydrogenation reactions of steroids, 746–748
  reduction, of $\Delta^4$double bond, 746
    of $\Delta^{16}$double bond, 747
    of side-chain aldehyde group, 748
    of side-chain ketone group, 747, 748
11$\alpha$-Hydroxyallopregnane-3,20-dione, 725, 746
1$\alpha$-Hydroxy-4-androstene-3,17-dione, 730, 737
6$\beta$-Hydroxy-4-androstene-3,17-dione, 725, 732
8-Hydroxy-compound S, 726, 735
14$\alpha$-Hydroxy-compound S, 726, 740
15$\beta$-Hydroxy-compound S, 726, 742
6$\beta$-Hydroxy-compound S, 733, 743, 744
14$\alpha$-Hydroxycortisone, 740

14α-Hydroxy-$\Delta^{9(11)}$-dehydro-compound S, 741
11α-Hydroxy-6-dehydroprogesterone, 736
11α-Hydroxy-16-dehydroprogesterone, 736
7α-Hydroxy-11-deoxycorticosterone, 734
14α-Hydroxy-11-deoxycorticosterone, 739, 740
15α-Hydroxy-11-deoxycorticosterone, 741
16α-Hydroxy-11-deoxycorticosterone, 742
17α-Hydroxy-11-deoxycorticosterone, 744
6β-Hydroxy-11-deoxycorticosterone, 732, 743, 744
15β-Hydroxy-11-deoxycorticosterone, 741
11β-Hydroxy-14α,15α-Epoxido-compound S, 738
ω-Hydroxyemodin, 524, 684, 693
14α-Hydroxyhydrocortisone, 725, 738, 740
Hydroxylation of steroids, 720–746
1α-Hydroxylation of steroids, 730, 731
6β-Hydroxylation of steroids, 731, 732
7-Hydroxylation of steroids, 733, 734
8-(or 9-)Hydroxylation of steroids, 734, 735
10-Hydroxylation of steroids, 735
11α-Hydroxylation of steroids, 735–738
11β-Hydroxylation of steroids, 735, 738
14α-Hydroxylation of steroids, 739–741
15-Hydroxylation of steroids, 741, 742
16α-Hydroxylation of steroids, 742
17α-Hydroxylation of steroids, 742–744
21-Hydroxylation of steroids, 745, 746
6-Hydroxy-2-methylbenzoic acid, 684
2-Hydroxymethylfurane-5-carboxylic acid, 525, 688
  formula, 688
6β-Hydroxy-17α-methyltestosterone, 732
11α-Hydroxy-17α-methyltestosterone, 737
6β-Hydroxy-19-nortestosterone, 732
10-Hydroxy-19-nortestosterone, 735
11α-Hydroxy-19-nortestosterone, 737
11α-Hydroxy-16α,17α-oxidoprogesterone, 737
-Hydroxyphenylacetic acid, 779, 780
1α-Hydroxypregnanedione, 736
6α-Hydroxypregnane-3,20-dione, 725, 742, 746
0β-Hydroxy-4-pregnene-2-one, 725, 747
β-Hydroxyprogesterone, 735, 745
1α-Hydroxyprogesterone, 724, 729, 736, 738, 739, 744, 746
1β-Hydroxyprogesterone, 738, 746
14α-Hydroxyprogesterone, 725, 739, 740
15α-Hydroxyprogesterone, 741
15β-Hydroxyprogesterone, 741
16α-Hydroxyprogesterone, 731, 732
17α-Hydroxyprogesterone, 743–745
11α-Hydroxy-17α-progesterone, 747
8-Hydroxyquinoline, 224
6β-Hydroxytestosterone, 732
11α-Hydroxytestosterone, 737
14α-Hydroxytestosterone, 741
16α-Hydroxytestosterone, 742
Hyphae, 515
Hypoxanthine, 241

Identification of yeasts, 32, 33
Ijichi, K., 484, 488, 489, 491, 492
Ilotycin, 798
Imai, K., 714, 716
Incubation cell, inclined (fig.), 851
*Industrial and Engineering Chemistry*, 886
Industrial microbiology, definition, 1, 3
  scope, 2, 3
Infectious hepatitis, 370
Ingelman, B. G. A., 370, 387, 388
Inoculation methods, 774–777, 849
Inoculation pan (fig.), 849
Inoculum preparation, 557–561
  method, Moyer, 558
    Steel, Martin, and Lentz, 558–561
Inosine, 241
Inosine triphosphate, 241
Inositol, 45, 61
Inskeep, G. C., 71, 74–77, 86, 314, 315, 317, 324
Interior view of fermentor (fig.), 785
Intermediate substances from yeast, 239-241
Invertase, 238, 245, 623
β-Ionone, 699
Ippen, A. T., 894
Iron lactate, 325, 326
Isinglass, 184
Islandicin, 525, 684, 693
Isoamyl alcohol, 43, 133, 142, 143
  formula, 43
  origin, 142, 143
Isobutyl alcohol formula, 44
Isocitric acid, 569
Isocitric dehydrogenase, 568–571, 721
Isolation, of molds, 520, 521
    by agar dilution, 520
    by germination of single spore, 520
    by Hansen method, 520
    by micromanipulator, 520
    by modification of Keitt single-spore method, 520, 521

Isolation, of molds, by picking spores from single spore head, 520
  and purification of antibiotics, 785, 786
Isolation methods, 28–32, 765
Isoleucine formula, 43
Isomaltose, 384
Isomerase, 615
Isoniazid, 797
Isopropyl alcohol, 277, 278, 285–293, 553
  effect on citric acid production, 553
Itaconic acid, 598–608
  applications and derivatives, 607, 608
  determination in fermentation liquors, 606, 607
  formula, 598
  production, 602–605
    fermentation for, 602–605
      changes in medium (fig.), 603
      data (table), 600, 601
      organisms used, 598, 599
    flow sheet, 604
    by submerged-culture method, 602–605
      method of Pfeifer and associates, 602–605
        data (table), 605
    by surface-culture method, 605
  properties, 598
  recovery, 605, 606
  references, 608
  yields, 602
Itatartaric acid, 525, 643, 688
  formula, 688
  production, 643
  reference, 643
Ito, S., 619
Ivashkevich, M. A., 504
Ivory soap, 558

Jackson, G. G., 796
Jackson, R. W., 379, 381, 382, 484, 886–888, 896
Jacobs, P. B., 113, 122, 872
Jacobs, W. L., 861, 862
Jamieson, G. S., 654, 655
Janicki, J., 483
Janke, A., 497
Jansen, H. E., 51
Jarvis, F. G., 770, 779
Javanicin, 684
Jawetz, E., 799, 804
Jeanes, A., 370–373
Jeffreys, G. A., 666–669
Jennison, M. W., 658
Jerusalem artichokes, 260, 306, 321, 322
  production from, of acetone, 260
    of L(+)-lactic acid, 321, 322
Johnson, M. J., 228, 244, 267, 271, 320, 322, 323, 403, 536, 546, 551, 561, 562, 572, 671, 770, 777, 779, 782, 887, 893–895, 898
Jones, A., 351
Jones, A. E., 388
Jones, E. R. H., 691
Jones, I. D., 344–348, 357, 358, 361,
Jones, K. L., 485
Johnston, M. R., 812
Jorgensen, A., 29
Joslyn, M. A., 46, 182, 239, 241, 242,
Josselyn, L. E., 798
Jukes, T. H., 484, 818
Jump, J. A., 115, 116, 855, 857, 858,
Jung, A., 698
Just, F., 72, 85

Kahnt, F. W., 724, 733, 736, 737
Kamen, M. D., 549
Kamlet, J., 465
Kane, I. H., 808
Kane, J. H., 579, 598, 602, 626–628
Kanel, E., 631
Kaolin, 677
Kaplan, N. O., 673
Karow, E. O., 546–550, 557, 562, 728, 72 887, 894–896
Karström, H., 478
Kastner, G., 446, 447
Katagiri, H., 305, 600–615, 714, 716
Kato, J., 620
Katznelson, H., 400, 405–407, 409, 41 414, 415
Kauble, H. B., 336
Keipper, C. H., 70, 73
Kelly, C. D., 337
Kelly, F. C., 250
Kempe, L. L., 307, 898
Kendall, A. R., 399, 401
Keresztesy, J. C., 226
3-Ketobisnor-4-cholene-11,22-diol, 748
3-Ketobisnor-4-cholene-11α,22-diol, 737
3-Ketobisnor-4-cholene-22-al, 748
Ketogenic activities of *Acetobacter* species, 466, 467
2-Ketogluconic acid, 506–508
  course of typical fermentation for, 50
  production by species of *Pseudomonas*, 506–508
  structural formula, 508
5-Ketogluconic acid, 462–464, 557
  composition of production medium, 463
  conditions of production, 463
  course of fermentation (fig.), 463
  organism used, 462
  patented process, 463, 464

5-Ketogluconic acid, preparation of inoculum, 462
  yields, 463
α-Ketoglutaric acid, 144, 569, 713, 714, 716–721
  conversion to L-glutamic acid, 718–721
    by enzymes, 720, 721
    by microorganisms, 718–720
    by *Pseudomonas ovalis,* 719
  formula, 713
  microorganisms producing, 714–716
  production, 713–718
    data, 717
    media, 717
    nutrients used, 716
    by *Pseudomonas fluorescens,* 716
    references, 721, 722
    some pathways for formation, 720
    yields, 717, 718
α-Ketoglutaric oxidase, 568–571
γ-Ketopentadecoic acid, 525, 684, 688
  formula, 688
11-Ketoprogesterone, 745, 746
Keussler, O., 109, 892
Kevorkian, 897
Kieber, R. J., 229, 232, 240
Kiessling, W., 142
Killey, M., 384
Killough, J. H., 798
King, P. S., 675
King, T. E., 469
King, W. S., 415, 422
Kinoshita, K., 598, 605, 609, 610, 613
  medium of, 610
Kinoshita, S., 721
Kirby, R. S., 814
Kirby, W. M. M., 809
Kirkpatrick, S. D., 806
Kita, D. A., 541, 713, 738, 752
Kitahara, K., 305, 610–615
Kitamura, H., 620
Kite, W. E., 186, 187, 189
Kiyomoto, U., 109
*Klebsiella pneumoniae* A.T.C.C. 10031, 794
Klein, E. F., 810
Klein, L., 609, 615
Kline, A. A., 873
*Kloeckera,* 34
  *apiculata,* 16, 17
Klose, A. A., 85
Kluyver, A. J., 131, 134–136, 273, 275, 301, 404, 428, 454, 510–512, 546, 896
*Kluyvera citrophila,* 714
    var. **α,** 714, 716–718
  *noncitrophila,* 714
Kluyver's scheme for alcoholic fermentation, 134–136
neen, E., 498–502, 860
Knight, B. C., 301
Knight, S. G., 770
Knudson, L., 638
Koepsell, H. J., 379, 381, 383, 713, 715–718
Koester, H., 752
Koffler, H. E., 770, 774
Kofranek, A. M., 622
Kohl, H. C., Jr., 622
Kohler, A. R., 810
Kojic acid, 609–618
  amount produced by *A. oryzae* (table), 612
  ferric chloride test for, 609
  fermentation, 609–616
    carbon sources for, 610
    concentration of carbon-containing substances used, 610
    duration, 611
    effect on, of added substances, 611
      of calcium carbonate, 611
      of chlorhydrin, 611
    history, 609, 610
    inhibition by acids, 611
    medium of Kinoshita for, 610
    molds producing, 609, 610
    nutrient salts for, 610, 611
    pH for, 611
    salts used by May and coworkers for, 610
    temperature, 611
  formula, 609
  mechanism of formation, 613–616
    Arnstein and Bentley, 615, 616
    Birkinshaw, Charles, Lilly, and Raistrick, 614
    Challenger, Klein, and Walker, 615
    Corbellini and Gregorini, 613
    Gould, 614
    Katagiri and Kitahara, 614
    Kinoshita and Haworth, 613
    May, Moyer, Wells, and Herrick, 613, 614
    Sakaguchi, 614
    Yabuta, 613
  microorganisms producing, 610
  properties, 609
  reactions, 611, 613
  references, 615, 617, 618
  uses, 613
  yields, 611, 612
Kojies, 676, 678, 679
  preparation, 678, 679
Kojima, N., 895
Kolachov, P., 88, 109, 837, 838
Kolfenbach, J. J., 422
Koniuszy, F. R., 484
Kooi, E. R., 414, 422, 464
Kooy, J. S., 813

Koppeschaar, W. F., 606
Kordecki, M., 896
Korenchevsky, 85
Kornberg, A., 240
Kozlow, D., 897
Krámli, A., 724, 733
Krampitz, L. O., 770
Krause, C. B., 157, 158
Krause, R. F., 698
Krausen, 162
Krebs cycle, 568, 569, 571
Kreger, D. R., 18
Kreger-van Rij, N. J. W., 10, 11, 14, 16, 28, 33–36, 72
Kroeker, E. H., 656
Kroll, C. L., 887, 897
Krueger, K. K., 307
Kulka, D., 428, 466, 468
Kurosawa, E., 619
Kurth, E. F., 27, 80–82, 85, 86
Kvass, 170

Lactalbumin, 309
Lactase, 236–238, 245
  production, 236, 238
  references, 245
Lactic acid, 304–331, 416–418, 420, 421, 630–636
  construction materials, 324, 325
  containers for distribution, 325
  derivatives, 326
  esters, 326
  forms, 304, 305
  grades, 311–313
  production, 304–331, 630–636
    by bacteria, 304–331
      from blackstrap molasses, 316
      carbohydrates suitable for, 306
      from casein whey, 308–311
        flow sheet, 310
      from cheese whey, 313
      commercial, 305–320
      concentration of sugar for, 306
      from corn sugar, 314–316
      fermentation, 309, 316
        acceleration, 322, 323
        continuous, 313, 314
        duration, 308
        mechanism, 326–328
        pH, 306, 307
        temperature, 306
      flow sheet, 315
      history, 304
      from Jerusalem artichokes, 321, 322
      neutralization of acid in, 306, 309
      organisms used for, 305, 306
      oxygen relationship in, 306
Lactic acid, production, by bacteria, potatoes, 316–318
      preparation of starter for, 309
      process in brief, 309
      references, 328–331
      from sulfite liquor, 320, 321
      yields, 308
    by molds, 630–636
      advantages, 635
      aluminum drum for, 634
      course of typical fermentation (fig.), 635
      effect on, of length of incubati period, 633
        of nitrogen sources, 631, 632
      history, 630
      methods, 630, 631
      references, 636
      respiration and, 635
      rotary-fermentor process, 634, 6
      surface-culture method, 631–634
      yields, 633–635
  publications of U.S. Dept. of Agricu ture on, 328
  purification, 323, 324
  references, 328–331
  uses, 325
Lactic acid bacteria, 63, 299–303
  classification, 300, 301
  data on reactions, 299
  nutritive requirements, 301
  references, 302, 303
Lactic dehydrogenases, 305
Lactobacillaceae, 165, 169
*Lactobacillus acidophil-aerogenes,* 301, 327
  *acidophilus,* 302
  *arabinosus,* 305, 483
  *brevis,* 305, 327, 334, 348, 349, 352, 356, 357, 365
  *bulgaricus,* 302, 306, 309, 313, 314, 397, 474
  *casei,* 306, 314, 320, 395, 475, 477, 483
  *cucumeris,* 333, 335, 337
  *delbrueckii,* 300–302, 305, 306, 318, 320, 322, 326
    NRRL B-445, 307, 676
  *japonicus,* 305
  *lactis,* 397, 494
  *leichmannii,* 265, 306
    A.T.C.C. 4797, 489
  *lycopersici,* 301, 327, 328
  *mannitopoeus,* 301
  *pastorianus,* 169
  *pentoaceticus,* 306
  *pentosus,* 305, 306, 365
    124-2, 307, 318, 321
  *plantarum,* 305, 333, 337, 349, 351, 352, 356, 357

Lactose, 306, 308, 476
  fermenting yeasts, 225, 226
Lafar, 428
Lager beer, 167
Lagoda, A. A., 886–888, 896
Lakata, G. D., 887, 898
Lang, A., 622
Langford, C. T., 380, 382, 386
Langlykke, A. F., 260, 287–289, 291, 292, 666, 804, 858, 859, 862
Lanolin, 621, 622
Lard oil, 594
Lardy, H. A., 673
Larson, E. J., 799
Lathrop, E. C., 260, 868–871
Laufer, L., 239–241
Laufer, S., 844, 853, 856
Lauric acid, 190
Lausten, O., 31
Lawrie, J. W., 209, 210
Leadbeater, E. F., 692
Lebedev, A., 130
LeBlanc, F. R., 813
Lechevalier, H. A., 484, 762, 763, 765, 798–800, 816
Lecithin, 26
Lederberg, E. M., 39
Lederberg, J., 39
Ledingham, G. A., 399, 401, 404–409, 412, 419–421, 897
Lee, C. C., 809
Lee, S. B., 897
Leeming, P. R., 691
Lees, T. M., 215
LeFevre, E., 332
Legg, D. A., 252
Leifson, E., 428, 430
Le Mense, E. H., 666, 858–861
Lemieux, R. U., 639–641
Lemoigne, M., 419
*Lentinus degener*, 694
Lentz, C. P., 546, 554, 556, 558–563, 569, 570, 887
*Lenzites abientina*, 732, 741, 755
Leonard, R. H., 118, 120, 260, 307, 320, 321
Leonhardt, H. G., 167
*Lepiota naucina*, 648
  *procera*, 648
  *rachodes*, 648
epper, M. H., 807
eslie, J. D., 399, 408–410
esser, M. A., 215
eucine, 42, 43
  formula, 42
*euconostoc citrovorum*, 510
  *dextranicum*, 510
  *mesenteroides*, 305, 333–336, 348, 349, 373
*Leuconostoc mesenteroides*, NRRL B-512, 379, 387
    NRRL B-512F, 372, 374, 382
      medium for, 382
    NRRL B-742, 387
Leucosin, 839
Levene, P. A., 129
Levin, A. P., 483
Levin, R. H., 724
Levine, J., 769, 787
Levine, M., 399, 404
Leviton, A., 484, 503, 504
Lewis, J. C., 85, 227, 813
Lewis, J. G., 484, 488, 489, 491, 492
Lewis, K. F., 568, 572
Lichens, 228
Liebmann, A. J., 64, 204, 422, 893
Lightfoot, E. N., 895
Lignin, 874–881
Lilly, C. H., 211, 614
Lilly, V. G., 698
Limit dextrinase, 862
Linaloöl, 153
Lind, H. E., 800
Lindegrin, C. C., 18, 22–25
Linder, W. V., 212
Lindner, P., 91
Lindner method, 30
Line filters, 77
Lines, G. O., 261
*Lipomyces*, 36
  *lipoferus*, 88, 89, 91
    fat production by, 91
  *starkeyi*, 14, 15, 89
*Lipomycetoideae*, 36
Lippincott, S. B., 607
Lochhead, A. G., 400, 414
Locke, E. G., 71, 78, 79
Lockwood, A. R., 387, 388
Lockwood, L. B., 457–459, 461–463, 465, 504–509, 599, 600, 602, 605, 625, 626, 631–635, 658–660, 671, 675–678, 713, 715
Lodder, J., 10, 11, 14, 16, 28, 33–36, 44, 72, 91
Loew, O., 93
Lohmann, K., 131, 142
Lohmar, R. L., 371
Long, P. H., 804
Long, R. A., 896
Longsworth, L. G., 898
Lovell, C. L., 421
Lovell, R., 770, 801
Lucas, E. H., 762
Lüdecke, K., 210, 211, 215
Luh, B. S., 238, 239
Lumb, M., 887, 894
Lundgren, D. G., 887
Lundin, H., 88, 90, 91

Lupulone, 153, 159
Luteic acid, 524, 695
Lutwak-Mann, C., 141
*Lycoperdon umbrinum*, 648
Lynen, E., 244
L-Lysine, 710–712
  commercial production, 710–712
  formula, 712

M solution for lipid production, 661
McAleer, W. J., 744, 745
McCandless, R. F. J., 650, 652, 653
McCann, E. J., 162–164
McCann, E. P., 896
McCleskey, C. S., 373
McCleskey, S., 399, 404
McClintock, M., 813
McCormick, M. H., 809
McCowen, M. C., 798
McCoy, E., 262, 291
McCready, R. M., 357
McCulloch, E. C., 452
McCutchan, W. F., 252
McDaniel, L. E., 770, 893, 896
MacDonald, F. J., 422
MacDonough, J. V., 86
McGhee, R. M., 374–376
McGuire, J. M., 798
MacInnes, D. A., 898
MacKin, J. J., 441
  process for making vinegar, 441, 442
Mackinney, G., 28
McLimans, W. F., 800
McMillan, G. E., 226
MacNaughton, J., 770
Madison wood-sugar process for wood saccharification, 877–881
  equipment, for neutralization of wood sugars (fig.), 880
    for saccharification of wood (fig.), 879
  modification, 881
Malchenko, A. L., 109
Malic acid, 524, 565, 567, 569, 688
  formula, 688
Malic dehydrogenase, 568–572
Malic enzyme, 568–570, 572
Malonic acid, 524, 688
  formula, 688
Malt, 150, 839–842
  barley for, 839–842
    composition, 839, 840
    germinated, drying of, 840
    germination, 841, 842
    selection, 839
    steeping, 840, 841
    storing, cleaning, grading, 840
  preparation, 839–842
Malt, uses, 842
Malt adjuncts, 150, 153
  treatment, 156, 157
Malt beverages, 148
  analyses, 149
  production by leading states, 150
Malt extract, 430
Malt medium, 519
Malt sprouts, 288, 301, 308, 314, 316,
    322, 413, 414, 430
Malt vinegar, 432
Maltase, 862
Malting plant (fig.), 839
Mamoli, L., 723, 746
Mancera, O., 724, 726
Mandels, G. R., 672
Mann, K., 738, 752
Mann, T., 141
Mannitol, 243, 333, 416, 455, 466,
    598, 644–646
  fermentation by *B. polymyxa*, 416
  formula, 645
  fructose production from, 466
  production, 644, 645
  references, 645, 646
Mannocarolose, 524, 695
D-Mannonic acid, 524, 688
  formula, 688
Mariller, C., 109
Marmell, M., 797
Marquardt, R. R., 84
Marth, P. C., 622, 623
Martin, S. M., 546, 553, 554, 556–56
    568, 569, 572, 887
Martin, W. R., 642
Marvel, C. S., 267
Mash cooking, batch-type pressure, 83
  continuous, 838
  infusion process, 837
Mash water composition, 157
Mashing methods, 153, 155–159
  decoction, 155, 156
  infusion, 153, 155, 156
Massengale, O. N., 229, 232, 240
Massock, H., 27
Masuo, E., 713, 714
Material balance in acetone-butanol fe
    mentation, 259
Materials of construction, 886
Matrishin, M., 484
Matromycin, 800, 801
Mattick, R., 813
Mattos, A. R., 109
Maxon, W. D., 887, 892, 893, 894, 896
May, O. E., 210, 539, 579, 581, 583, 59
    610, 611, 613, 626, 631–633, 658–660
Maze, P., 563
Mazola oil, 221
Meade, R. E., 503, 504

Meara, M. L., 656
Mechanism, of ethyl alcohol fermentation, 141
  of fermentation, methods of studying, 129–131
Meder, H., 625, 626
Mehler, A. H., 673
Meister, P. D., 732, 736, 737, 740–742, 744, 746, 748
Melibiase, 237
Melibiose, fermentation, 37
  formula, 37
Melle process, 118
Mellein, 696
  formula, 696
Mellors, H., 896
Mendlik, F., 51
Menzinsky, G., 61, 62, 70
Menzshinskaya, E. W., 567
Merck and Company, 482, 786, 801
Merritt, P. B., 70
Merten, J. A., 167
Metabolic products from molds (table), 522
Methanol, 323, 324, 377, 378, 384–386
Methionine contents of yeasts, 227, 228
Methods of saccharifying starch-containing materials, 836, 837
-Methoxy-2,5-toluquinone, 684, 694
  formula, 694
lethyl alcohol effect on citric acid production, 553–555
  (*See also* Methanol)
ethylanisate, 684
ethylcinnamate, 684
ethyldiethylarsine, 697
Methyl glucoside, 384
ethylglyoxal, 132, 136, 695
  hydrate, 135, 136, 276
ethyl lactate, 316, 318, 324
ethyl *p*-methoxycinnamate, 684
ethyl Red-Voges-Proskauer medium, 406
Methylsalicylic acid, 524, 689
  formula, 689
γ-Methyltetronic acid, 524, 683, 684, 689
  ormula, 689
thylene blue, 453
thylene chloride, 727, 728
thylene succinic acid (*see* Itaconic acid)
yerhof, O., 129, 137, 138, 140, 142, 572
ystre, C., 732–734, 741, 742, 744–747, 751, 752
haelis, L., 129
hener, H. D., 813
robial amylase preparations sources (table), 857
Micrococcaceae, 165
*Micrococcus flavus* A.T.C.C. 10240, 794
Micromanipulator method, 30
Microorganisms, of fermentation, 4
  synthesizing riboflavin, 219
*Microsporon audouini*, 519
  *lanosum*, 519
Mikeska, L. A., 607
Milk, 393
Miller, E. S., 114
Miller, I. M., 800
Miller, J. J., 31
Miller, S. A., 895
Miller, T., 493
Miller, W. H., 810
Millet, 503
Mills, W. D., 814
Milner, R. T., 485
Mineral requirements of yeasts, 45–47
Minioluteic acid, 524, 683, 684, 689
  formula, 689
Mirimanoff, A., 221
Miso, 678
Mitchell, J. A., 301
Mitchell, J. E., 620
Mitchell, J. W., 814
Mitson, G. W., 430
Miyaji, K., 630
Mizaume, 526
Modern beer storage cellar (fig.), 162
Molasses, treatment, 67
Molasses-ammonium process for yeast, 65–69
Molasses-corn-steep medium, 230
Mold bran, 844–858
  apparatus for growing (fig.), 847
  development of process, 844, 845
  vs. malt as saccharification agent, 854, 855
  molds used, 845
  production methods, 846–852
    commercial, 852
    laboratory, 846, 847
      drum, 846
      pot, 846, 847
      tray, 847
    pilot-plant, 848–850
    semicommercial, 850–852
      flow sheet (fig.), 852
Mold enzymes, 666–675
  cellulose from *Myrothecium verrucaria*, 672
  charts, 674, 675
  continuous tray method for producing, 666–669
    drying and bagging, 669
    handling of trays, 669
    preparation and inoculation of substrate, 668, 669

Mold enzymes, continuous tray method for producing, preparation, and inoculation of substrate, 668, 669
      of inoculum, 668
    traying and incubation, 669
  location, 672
  pentosan breakdown, 672
  proteases from *Mortierella renispora,* 671, 672
  references, 679–687
    general, 673
  submerged culture methods, 666
  from *Trichoderma koningi,* 672
  uses, 672–675
Mold fat content, 657
Mold pigments, formulas, 693, 694
Mold polysaccharides, 694, 695
  produced from glucose, 695
Mold preparations as conversion agents, comparison with malt, 855–857
Molds, 515–532
  acids of, 524, 525
  *Aspergilli,* important, 526
  classification, 516
  coenocytic, 515
  composition (table), 657
  containing more than 15 per cent crude fat (table), 658
  cultivating apparatus, 530
  divisions of penicillia, 526, 528
  enzymes (table), 524, 525, 529
  general characteristics of *Rhizopus,* 528
  general description, 515, 517
  growth media, 518, 519
    Czapek's, 519
    malt, 519
    natural, 519
    Raulin's, 518
    Sabouraud's, 519
  growth method, 515
    apical, 515
    intercalary, 515
  growth requirements, 517, 518
    carbon sources, 518
    essential elements, 517
    nitrogen sources, 517
    other elements, 518
  identification, 521, 522
  important genera, 523, 527
  isolation methods, 520, 521
  lipid content, 655–658
  pigments, 684, 693, 694
  polysaccharides of, 694, 695
  products (table), 524, 525
  references, 529–532
  some mold metabolic products (table), 522
  sterols, 229, 656, 657
  subdivision of *Penicillium* (fig.), 527
Molecular filter, 383
Molinari, E., 452
Mollenhauer, H. P., 448, 449
Mollet, H. W., 121
Molliard, M., 578
Molybdenum, 537
*Monilia formosa,* 642, 686
Monoacidic beta-cellobiolipids, 6
Monod, J., 892
Monoiodoacetic acid, 130, 142
*Monosporella,* 36
Monoverticillata, 526, 527
*Morchella conica,* 653
  *crassipes,* 648, 653
  *esculenta,* 653
Morel mushrooms, 653
Morgan, E. R., 238
Morgen, R. A., 73, 79
Morikawa, K., 285, 291, 679
Morris, M., 85
*Mortierella renispora,* 671–673
  proteases, 671, 672
Mosbach, E. H., 572
Mowry, D. T., 607
Moyer, A. J., 539, 546, 553–555, 55
    562, 579–590, 593, 599, 600, 60
    626, 643, 770, 773, 774, 777, 77
    780
Mrak, E. M., 10–13, 31, 33
*Mucor boulard* No. 5, 864
  *corymbifer,* 755
  *griseocyanus,* 739, 740, 750, 755
  *mucedo,* 697, 755
  *parasiticus,* 735, 750, 755
  *piriformis,* 533, 534
  *racemosus,* 695
  *rouxii,* 630, 864
  *varians,* 713
Mulholland, T. P. C., 619, 620
Multipoint inoculator, 40
  illustration, 41
Mundt, J. O., 351
Munns, D. J., 61
Murphy, D., 543
Murphy, J. R., 215
Murray, H. C., 724, 733–735, 739, 75
Murray, S., 659
Mushroom mycelium, 647–653
  B-vitamins of, 653
  composition of, 652
  flavor of, 651, 652
  media for, 649, 650
  nutritive value of, 652, 653
  production, 647–651
    on different media, 652
    fungi for, 647, 648
    methods, Block and associates, 652
      Humfeld and Sugihara, 647–65

Mushroom mycelium, references, 653
  yields, 648
Musselman, M. M., 808
Mustakas, G. C., 422
Mustard oil, 353
Mutschler, W. J., 162
Mycelin, 817
Mycelium, 515
*Mycobacterium phlei*, 483
  *smegmatis*, 483
  *tuberculosum*, 483, 710
Mycoderma, 352, 353
Mycodextrin, 524, 695
Mycomycin, 817
Mycophenolic acid, 524, 689
  formulas, 689
Mycostatin (*see* Nystatin)
Mycothricin, 817
Myers, R. P., 236
Myo-inositol, formula, 48
Myrbäck, K., 673
Myrcene, 153
*Myrothecium verrucaria*, 672
  cellulase of, 672

*Nadsonia*, 36
  *elongata*, 14, 15
*Nadsonieae*, 35
Nagase, H., 716
Nägeli, C., 93
akayama, T., 28
alcite AX, 224
alcite iron remover, 224
alcite MX, 224
algiolaxin, 525, 684, 693
algiovensin, 525, 684, 693
aphthaquinone derivatives, 684
aphthosulfonate indophenol, 131
athan, F., 251
ational Research Council of Canada, 400
atural media, 519
ear-synthetic medium, 229, 230
eedle, H. C., 316, 319
eilands, J. B., 673
eish, A. C., 399–401, 419–422, 572
lson, E. N., 465
lson, G. E. N., 504, 508, 509, 600–602, 605
lson, H. A., 887
lson, M. E., 326–328
*matospora*, 36
  *oryli*, 14, 15
*matosporoideae*, 36
ashev, P. K., 797
omycin, 794, 799
eferences, 820
berg, C., 26, 238, 273, 472
Neuberg ester, 129
  formula, 132
Neuberg's scheme for alcohol fermentation, 131–134
*Neurospora crassa*, 229, 720, 735, 756
New Brunswick shaker, 243
Newton, H. P., 113, 122, 872
Ni, S. M., 678
Nichols, R. S., 546
Nickerson, W. J., Jr., 36
Nicotinic acid, 27, 48, 227, 305, 307
  formula, 48
Nielsen, J. K., 806
Nielsen, N., 42, 88
Nienhuis, A. L., 359
Nilsson, N. G., 88
Nisin, 762, 809, 810, 812
  preservative action, 812
Nitrogen-containing compounds in yeast, 25
Niven, C. F., Jr., 301
Nobile, A., 748
Noble, W. M., 85
*Nocardia acidophilus*, 692, 817
Nøhr, B., 51
Nöllner, C., 474
Nolte, A. J., 81
Nondialyzable enzymes, 141
Nord, F. F., 695
Nordsiek, F. W., 70
Nordstrom, L., 388
Norit, 310, 311, 312
19-Nor-11-deoxycorticosterone, 746
19-Norprogesterone, 746
Northcote, D. H., 18
Northern Regional Research Laboratory, 643, 675, 801
Northern Utilization Research and Development Division, 219, 379, 382, 485, 598, 599, 642, 844, 845, 859, 868, 888
Northrop, J. H., 295, 297, 298, 896
Nortje, B. K., 357
Novak, L. J., 389
Novobiocin, 794, 799, 800
  formula, 799
  references, 820, 821
Nowell, W., 219
Nucleosidase, 237
Nucleotidase, 237
Nucleotides, 240
Nutrition of yeasts, 40–49
Nutritive value of food yeast, 85, 86
Nystatin, 794, 800, 819
  references, 821
N-Z-amine, 230
N-Z-amine A, 485
N-Z-amine B, 808

Oat hulls, 296, 298, 663
Oats, 503
Obayashi, A., 305
O'Brien, R. T., 813
Octadecanol, 620
  in ethanol, 603
  in lard oil, 780, 781
  (*See also* Octadecyl alcohol)
Octadecyl alcohol, 506
O'Flaherty, F., 654
Ohmer, H. B., 355, 356
Oishi, K., 713
Okumura, S., 714, 716
Oleandomycin, 800, 801
Oleic acid, 507
Oligomycin, 815
Olive, T. R., 308–310, 323
Olive oil, 221, 223
Olives, pickled green, 363–365
Olson, B. H., 228, 403
Olson, W. J., 860
Omelianski, V. L., 239
Onaga, D. M., 678
O'Neill, H. T., 659
Oösporein, 525, 684, 694
  formula, 694
*Ophiobolus herpotrichus,* 745, 746, 751, 752, 756
Ordal, Z. N., 88–91
Orla-Jensen, S., 301, 307, 474, 475
Orleans process, 438
Ormandy, W. R., 872
Osburn, O. L., 285, 286, 290–292
Oschatz double-drum driers, 79
Osmophilic yeasts, 242–244
Osnizkaya, L. K., 536
Ostern, P., 240
Oswald, E. J., 806
Othmer, D. F., 422
Otsuka, S. I., 716, 718, 719
Otte, N. C., 301
Ottke, R. C., 229
Owen, S. P., 892
Owen, W. L., 109
Owens, H. S., 357
Oxalic acid, 524, 536, 537, 541, 543, 544, 549, 550, 557, 689
  effect of pH on formation, 537
  formula, 689
Oxalosuccinic acid, 569
Oxford, A. E., 229, 542, 543, 598, 605, 606
Oxidoreductase, 136
Oxidoreduction reactions, 134, 135
Oxyjavanicin, 684
Oxytetracycline (terramycin), 807–813, 815, 818
  formula, 807
  use as food preservative, 809–811

*Paecilomyces divaricatum,* 534
  *variota* 1, 657, 658
  *variota* NRRL 1282, 642
Pagano, J. F., 800
Page, A. C., Jr., 484
Paladino, S., 887, 889
Pan, S. C., 88, 322, 323
Pantothenic acid, 27, 48, 227, 307
  formula, 48
Paradís, P. M., 601
Parker, A., 896
Parker, L. F. J., 482
Parnas, J. K., 142
Pasternack, R., 465, 466
Pasteur, L., 428, 431, 432
Pasteurization, of beer, 163
  of pickles, 355, 356
Patulin, 813
Patzelt, G., 229, 232
Pautard, F. G., 388
Payne, W. J., 229, 232, 240
Peanut hulls, 296, 298
Peanut oil, 221
Pearl, I. A., 770
Pearson, L. K., 654
Peckham, G. T., Jr., 308
Pectic acid formula, 359
Pectin formula, 359
Pectinesterase, 357, 673
Pectolytic enzymes, 186
Pederson, C. S., 301, 333–337, 34 420
Pederson, R. L., 735, 737
*Pediococcus cerevisiae,* 301, 348, 34 356, 357, 804
Peel, J. L., 239, 242
Pellet, 559
  effect on development, of ferroc and pH, 559
    of ferrocyanide and spore leve
    of pH and spores, 561
Peltier, G. L., 498, 499
Penicillic acid, 524, 684, 689
  formula, 689
Penicillin, 801–804, 813, 815
  assay methods for, 786, 787, 789–
  chemical structure of (table), 803
  historical, 801
  production, 770–785
  properties, 802, 803
  references, 821–827
  types, 803, 804
Penicillin F, 803, 804
Penicillin G, 780, 803, 804
Penicillin K, 779, 780, 803
Penicillin X, 803
Penicillinase, 502, 769
  action on Synnematin B, 806
*Penicillium adametzi,* 751

*Penicillium aurantio-brunneum*, 655, 656
  lipids of, 655, 656
 *aurantio-virens*, 690
 *brefeldianum*, 687
 *brevicaule*, 697
 *brevi-compactum*, 229, 686
 *camemberti*, 522, 528
  some enzymes produced by, 529
 *capreolinum*, 695
 *charlesii* G. Smith, 683, 685, 695
 *chrysogenum*, 579, 581, 593, 644, 669, 687, 756, 771–774, 778
 *chrysogenum* Wisconsin Q176, 778, 782
 *cinerascens*, 686, 694, 696
 *citreo-roseum*, 693
 *citrinum*, 534
 *corylophilum*, 739
 *crateriforme*, 690
 *cyclopium*, 689, 693
  var. *album*, 686
 *daleae*, 610
 *digitatum*, 527, 528, 695
 *expansum*, 528
 *flexuosum*, 687
 *funiculosum*, 688
 *glaucum*, 720
 *griseo-fulvum*, 626, 687, 799
 *hennebergi*, 305
 *javanicum*, 88
  fat production by, 659, 660
  lipids of, 654, 655
 *Johannioli*, 690
 *islandicum*, 693
 *italicum*, 527
 *lilacinum*, 88, 387, 656, 739, 751, 756
  fatty acids of, 656
 *luteum*, 533, 534, 695
 *luteum-purpurogenum*, 527
 *minio-luteum*, 689, 690
 *nalgiovensis*, 693
 *nigricans*, 799
 *notatum*, 699, 777, 800, 801
 *notatum-chrysogenum* group, 765, 766
 *obscurum*, 696
 *patulum*, 696
 *phoeniceum*, 694
 *pinophilum*, 527
 *puberulum*, 229, 689
 *purpurogenum* var. *rubrisclerotium*, 688
 *esticulosum*, 669
 *oqueforti*, 397
 *oseo-purpureum*, 693
 *ubrum*, 694
 *ugulosum*, 695
 *clerotiorum*, 695
 *oppi*, 88
 *piculisporum*, 688, 690

*Penicillium spinulosum*, 88, 658
 *stipitatum*, 690
 *tardum*, 739
 *Terlikowskii*, 696
 *terrestre*, 690
 *urticae*, 799
 *variabile*, 695
 *variens*, 695
 *vermiculatum* NRRL 1009, 642
 *viniferum*, 686
 *westlingii*, 229
Penicillus, 527
 diagram, 528
Pentagalacturonic acid, 239
Pentonic acids, production by *Pseudomonas* species, 508, 509
Pentosan breakdown, 672
Pentosan hydrolysis, 868, 869
Perithecia, 526
Perlman, D., 403, 536, 541, 542, 545–547, 561, 742, 746, 762, 770, 897
Peroxidase, 623
Perquin, L. H. C., 545–547, 557
Perrier, A., 563
Perseitol, 455
Perseulose, formula, 461
 production, 461
Pervozvanskii, V. V., 504
*Pestalotia foedans*, 736, 756
 *funera*, 756
Peters, R. A., 85
Peters, W., 768
Peterson, D. H., 723, 724, 727, 729–737, 739, 742, 746–748, 751, 752, 760
Peterson, M. S., 301
Peterson, W. H., 26, 27, 73, 79, 80, 227–229, 232, 260, 267–272, 288, 289, 291, 296–298, 301, 307, 308, 320, 322, 323, 326, 332, 466, 467, 474, 476, 541, 655, 657, 661, 671, 770, 778, 779, 887, 896, 897
Petsiavus, D. N., 222, 728, 729
Pette, J. W., 813
Pettijohn, O. G., 401–403
Petty, M. A., 484
Peyros, E., 538
Pfeifer, V. F., 219, 220, 252, 484–488, 601–606, 887
pH control, references (table), 898
Phaff, H. J., 12, 13, 28, 33, 36, 39, 40, 238, 239, 358, 359
Phares, E. F., 572
Phenylacetamide, 779
Phenylacetic acid, 770–780
Phenylalanine formula, 43
Phenylethyl alcohol formula, 43
β-Phenylethylamine, 779
Philip, J. E., 804
Phillips, G. E., 85
Phillips, G. F., 351

- Phlobaphene, 160
- Phoenicin, 525, 684, 694
  - formula, 694
- Phosphate effects on citric acid production, 554, 556, 557
- 2-Phosphoglyceric acid, 132, 138, 140–142
  - formula, 132
- 3-Phosphoglyceric acid, 132, 138–142
  - formula, 132
- Phosphopyruvic acid, 138, 141, 142
  - formula, 138
- Phosphorus, 46
- Phosphorylase, 237
- Phosphorylation, 137
- *Phycomyces blakesleeanus,* 698, 733, 741, 756
- Physcion, 525, 684, 693
- Phytochemical reduction, 51, 52
- *Phytophthora phaseoli,* 815
- *Pichia,* 35
  - *alcoholophila,* 228, 351
  - *membranaefaciens,* 14, 15, 83
- Pickled green olives, 363–365
  - packaging, 365
  - production, 363–365
    - grading, 364
    - harvesting, 364
    - lye-treated, brine treatment, 364
      - fermentation, 364, 365
      - washing, 364
    - selection of suitable varieties, 363
- Pickles, 341–369
  - brine fermentation, 344–349
    - changes, 344–349
      - biochemical, 345, 346
      - microbiological, 346–349
    - control of scum, 352, 353
    - use of sorbic acid, 351, 352
    - yeasts associated with, 349–353
  - classification, dill, 341
    - sour, 341
    - sweet, 341
  - cucumbers for, 342
    - grading, 342
    - influence of salt, 344
    - salting, 343
  - definition, 341
  - grading, 353
  - microbiological examination of, 361
  - pasteurization, 355, 356
  - preparation, 353–355
  - preservation-prediction chart, 360
  - processing of salt stock, 353
  - references, 365–369
  - spoilage, 352, 353, 356–361
    - bloater formation, 356–357
    - softening, 357, 358
- Pierce, J. V., 484
- Piersma, H. H., 222
- Pigments, 28, 524, 525, 684, 693, 694
  - of molds, 693, 694
  - some derivatives produced by m
    - anthraquinone, 684
    - toluquinone, 684
  - of yeasts, 28
- Piret, E. L., 897
- Pirie, N. W., 240
- *Pityrosporum,* 34
  - *ovale,* 16, 17
- Plant disease control with antibiot 812, 814, 815
- Plastic covers for tanks, 336
- Pledger, R. A., 800
- *Pleutotus ostreatus,* 648
- Plow, R. H., 872
- Podbielniak extractor, 785
- Pohler, G. M., 896
- Poisons, selective, 130
- "Polidase-C," 847
- Pollard, H. L., 503
- Polotsky, A. Z., 563
- Polyethylene, 378
- Polygalacturonase, 238, 239, 357, 359, 6
  - reactions on pectic acid, 238
  - references, 245
- Polyglucosans, 370
- Polyhydric alcohols, 242–244
  - production by osmophilic yeasts, 242 244
  - references, 246
- Polymyxin B, 762
- Polymyxins, 794, 804
  - references, 827
- Polypectate gel, 357
- Polypeptidase, 237
- Polyporic acid, formula, 689
- *Polyporus anthracophilus,* 691, 692
  - *fumosus,* 692
  - *nidulans,* 689
  - *officinalis,* 685
  - *sulphureus,* 648
  - *tumulosus,* 687, 691
- Polysaccharides of molds, 524, 695
- *Polyverticillata-symmetrica,* 526, 527
- Polyvinylpyrrolidone, 370
- Pombe, 171
- Pontillon, C., 654
- Pool, E. L., 666, 858, 860, 862
- Porges, N., 459, 536, 538, 579, 580, 582–588, 593
- Porter, R., 399, 404
- Porter, R. W., 806
- Porzecanski, B., 802
- Potassium ferrocyanide, 542–544, 552, 553, 559, 562
  - effect on growth of *A. niger* and citric acid production, 552, 553

Potatoes, 316, 318
  ethyl alcohol from, 113
  lactic acid from, 316, 318
    yields of, with different species of *Lactobacillus*, 318
Pottevin, H., 637
Poultry preservation with antibiotics, 810
Pozen, M. A., 158
Prange, C. H., 607
Pratt, R., 765, 770, 777, 778, 784, 785, 802, 804
Prednisone, 748, 749
Preece, I. A., 171
$\Delta^{1,4}$-Pregnadiene-11$\beta$,17$\alpha$, 21-triol-3,20-dione, 748
$\Delta^{1,4}$-Pregnadiene-17$\alpha$, 21-diol-3,20-dione, 749
$\Delta^{1,4}$-Pregnadiene-11$\beta$, 21-diol-3,20-dione, 748, 749
$\Delta^{1,4}$-Pregnadiene-21-0l-3,20-dione, 21-acetate, 748, 749
$\Delta^{4}$-Pregnene-17$\alpha$,20$\beta$,21-triol-3-one, 724, 726, 747
Prescott, F. J., 594
Prescott, S. C., 285, 533–535, 537–542, 546, 605, 784
  medium citric acid, 535
Preservation, fish, 810, 811
  fresh meat, 811, 812
  poultry, 810
Preservation-prediction chart, 360, 361
Pricer, W. E., Jr., 240
Prickett, P. S., 229, 240
Pridham, T. G., 219, 223, 762
Prigot, A., 797
Prill, E. A., 661
Primack, N., 806
Primer, 383–385
Pringsheim, H. H., 285
*Proactinomyces erythropolis*, 756
  *roseum*, 733
Proctor, B. E., 10
Products, of bacterial fermentation, 249
  formed, by molds (table), 524, 525
    by yeasts or yeast-like microorganisms, 51, 52
Progesterone, 725, 728, 729, 732, 736, 738–746, 751, 752
  some steroids produced from (fig.), 725
roof, definition, 102
ropagation, 6
  yeast, 60–86
    bakers', 60–70
    food and fodder, 71–86
    references, 95–99
*opionibacterium arabinosum*, 475
  *freudenreichii*, 475, 483, 484
  *jensenii*, 475
  *pentosaceum*, 475
*Propionibacterium peterssonii*, 475
  *raffinosaceum*, 475
  *rubrum*, 475, 476
  *shermanii*, 397, 475, 477, 483
  *technicum*, 475
  *thoenii*, 475, 476
  *zeae*, 475, 483
Propionic acid, 272, 292, 474–481
  bacteria, 475
    growth factors, 476
    media for, 476, 477
    sources, 475
      carbon, 475
      nitrogen, 475, 476
  fermentation, 477
    duration, 476, 477
    history, 474
    mechanism, 477–479
    pH, 476
    products, 477
    temperature for, 476
  ratio to acetic acid, 477
  references, 479–481
  uses, 477
  yields, 477
Propylene glycol, 510–512, 725, 728
$\alpha$-Propylene glycol formula, 455
*Proteus mirabilis*, 475
  *vulgaris*, 483, 484
Prouty, C. C., 301
Pruess, L. M., 229, 232, 307, 644, 657, 658, 661
Pseudomonadaceae, 165, 168
*Pseudomonas aeruginosa*, 507, 804, 805
  *fluorescens*, 506, 507, 509, 814
  *fluorescens* NRRL B-334, 720
  *fluorescens (reptilivora)*, 715, 716
  *fragi*, 507, 509
  *graveolens*, 507–509
  *mildenbergii*, 507, 509
  *mucidolens*, 505
  *myxogenes*, 505
  *ovalis*, 506, 507, 509, 719
  *pavonacea*, 507
  *phaseolicola*, 814
  *putida*, 507, 509
  *putrifaciens*, 509
  *schuylkilliensis*, 507
  *striafaciens*, 505
  *synxantha*, 509
  *syringae*, 505
  *tabaci*, 814
  *vendrelli*, 507, 509
Pteroylglutamic acid formula, 48
Puberulic acid, 524, 684, 690
  formula, 690
Puberulonic acid, 525, 684, 690
  formula, 690

Pulley, G. N., 81
Pulque, 170, 171
Pumice stone, 435
Puromycin, 763
Purpureo-cobaltic chloride, 609
Putnam, L. E., 808
Pyridones, 611
Pyridoxal, formula, 48
Pyridoxal phosphate, 721
Pyridoxamine, formula, 48
Pyridoxine, 27, 227
Pyridoxine hydrochloride, 45, 48
  formula, 48
Pyrogallol, 153
Pyrrolidone carboxylic acid, 712
Pyruvic acid, 132, 135, 139, 141, 142, 272, 292, 326–328, 416–418, 525, 563, 569
  fermentation, by *Aeromonas hydrophila,* 418
    by *B. polymyxa,* 416
  oxidase, 568–570

Quick vinegar process, 439
Quilico, A., 536
Quinic acid, 475, 566
Quinols, 51
Quinones, 51

R salts, composition, 379
Racking of wines, 183
Raffinose fermentation, 37
Raffy, A., 219, 221
Rahn, O., 352
Rainbow, C., 430
Raistrick, H., 229, 563, 598, 605, 606, 614, 626, 644, 645, 683, 695, 770, 801
Rake, G., 802
Ramakrishnan, C. V., 568–571
Ramaswamy, S., 229
Randall, W. A., 769, 787, 794–796
Rankin, J. C., 372
Rao, M., 430, 431
Raper, H. S., 654
Raper, K. B., 521, 526, 599, 697, 762, 765–767, 770, 772
Rapid conversion of mash, 842, 843
  effect of conversion times on fermentation yields (table), 843
Rappaport, L., 622
Ratios of neutral solvents, 253–255
Rattan, 436
Raulin's medium, 518
Ravenelin, 525
Ray, S., 352
Raymond, A. L., 129
Redfern, S., 836, 862
Reed, D. L., 422
Reed, G., 673
Reeves, M. D., 599, 600, 605
Reeves, R. V., 808
Regna, P. P., 805, 808
Reichstein's compound S, 724, 728, 734, 738, 740, 743, 745, 747, 748, 7
  steroids formed from (fig.), 726
Reichstein's substance U, 748
Reid, D., 801
Reilly, H. C., 770
Reilly, J., 272
Reiser, C. O., 83
Rendiconti Istituto Superiore di Sa 886
Rennet, 393
Replacement medium, 547, 549
  for citric acid production, 549
Replica-plating technique, 39
  illustration, 40
Respiratory coefficient, 842
Rettger, L. F., 263
rH, 160
Rhamnose, 510, 511
  fermentation by *Bact. rhamnosiferm tans,* 510, 512
  formula, 511
Rheinau process (modified) for wo saccharification, 881, 882
*Rhizopus arrhizus,* 630, 728, 731–734, 7 756
  *cambojae,* 731, 732, 757
  *chinensis,* 630, 631
  *delemar,* 522, 528, 845, 864
  *elegans,* 631
  *japonicus,* 522, 528, 626, 630, 864
  *necans,* 528
  *nigricans,* 522, 528, 529, 625–628, 72 732, 735–737, 746–748, 751, 752, 7
    diagram, 529
  *niveus,* 626
  *nodosus,* 528
  *oryzae,* 523, 528, 626, 630–636, 845
  *pallida,* 699
  *pseudochinensis,* 626, 630
  *reflexus,* 732, 757
  *salebrosus,* 631
  *sannieli,* 698
  *shanghaiensis,* 626, 630
  *stolonifer,* 631
  *tonkinensis,* 864
  *tritici,* 626, 631
Rhodes, A., 815
*Rhodotorula flava,* 698, 699
  *glutinis,* 16, 17, 26, 31, 88–91, 100, 101 227, 228
    amino acids of, 90, 91
    fat production by, 88–91
  *gracilis,* 698, 699
Rhodotoruloideae, 34

Riboflavin, 27, 45, 49, 219–227, 232–234, 307, 503, 504
  microorganisms producing, 218, 219
  production, 218–225
    by *Ashbya gossypii,* 219–221
    by bacteria, 503, 504
    by *Candida* species, 222–224
    by *Eremothecium ashbyii,* 221, 222
    flow sheet, 220
    by lactose-fermenting yeasts, 225–226
  properties, 218
  recovery, 225, 226
  references, 232–234
  structural formula, 218
D-Ribonic acid, 508, 509
Ribonuclease, 237
Ribonucleic acid, 240
D-Ribose, 33
Rice, 263
Richards, M., 710
Richards, O. W., 47
Richardson, A. P., 802
Rickes, E. L., 482, 484
Riedl-Tumova, E., 723
Rimocidin, 815, 817
Rist, C. E., 371, 372
Ristocetin, 804
  references, 827
Ritter, G. J., 260, 868
ivett, R. W., 671, 887
obbins, E. D., 352
oberts, I. S., 238
oberts, M., 847, 853, 854, 856
oberts, O., 543
oberts, R. B., 721
obertson, A., 695
obertson, F. M., 421
obinson, R. C. V., 800
obinson, R. S., 814
obison, R., 129
obison ester, 129, 134
  formula, 132
obitzek, E. H., 797
ockwell, G. E., 654
odgers, N. E., 503
oe, E. T., 457–459, 461–463, 504, 506, 508
elofsen, P. A., 18
gers, L. A., 308
gers, S. C., 84, 118, 120
gosa, M., 109–112, 225, 226, 301
govin, S. P., 380, 382, 386
jowski, P., 88, 90
inson, G. N., 894
mansky, M. J., 804
uefort cheese-making, 397
e, D., 399, 415–417, 422
eopurpurin, 525, 684, 693
indulin GG, 131

Rotary drum fermentor, 580–586
  laboratory-scale apparatus (fig.), 580
  plan (fig.), 586
Rotaventin, 817
Roth, E. R., 895
Routien, J. B., 765
Roxburgh, J. M., 244, 639–641, 887
Rubbo, S. D., 546
Ruben, S., 549
Rubin, B. A., 738, 745
Rubrofusarin, 525
Rudert, E. J., 221
Ruger, M. L., 484
Rugulose, 524, 695
Rum, 196–200, 205, 206
  composition, 197, 198
  definition, 200
  government supervision, 200
  production, 198–200
    aging, 199, 200
    distillation, 199
    fermentation, 198, 199
    regions of, 196, 197
  references, 205, 206
  types, 197, 198
Ruppol, E., 232
Russel, R. T., 887
Russell, C., 301
Rutgers University, 805
Rye, 503
Rye whiskey, 201

Sabouraud's medium, 519
Saccharic acid, 563, 564
Saccharification of mashes, 852–854
  methods, commercial-scale, 853, 854
    Hao, Fulmer, and Underkofler, 852, 853
    Roberts, Laufer, Stewart, and Saletan, 853
Saccharifying agents, 836–885
*Saccharomyces anamensis,* 226, 864
  *carlsbergensis,* 14, 15, 25, 38, 83
  *cerevisiae,* 14, 15, 18, 20, 22, 71, 72, 74, 198, 201, 211, 227, 228, 230–232, 236, 720, 843
  *cerevisiae* A.T.C.C. 9763, 794
    var. *ellipsoideus,* 182, 434
  *fragilis,* 226, 236, 238, 720
  *globiformis,* 350, 360
  *globosus,* 350, 351
  *halomembranis,* 350–352
  *lactis,* 226, 236
  *mellis,* 14, 15
  *oviformis,* 232
  *pastori,* 350
  *pastorianus,* 164, 166
  *pyriformis,* 171

*Saccharomyces rouxii,* 244
  *tokyo,* 171
  *validus,* 25
  *yeddo,* 171
Saccharomycetaceae, 10, 14, 15, 33, 35
*Saccharomycetoideae,* 35
*Saccharomycodes,* 36
  *ludwigii,* 14, 15
Saeman, J. F., 72, 78, 79, 84, 872, 873, 887, 897
Safflower, 699
Saha, K. C., 543
Saito, E., 630
Saito, K., 609
Sak, S., 892
Sakaguchi, K., 626, 643
Sakaguchi, K. I., 714, 716
Sake, 171, 678
Salinometer, 361, 363
  salt percentages and corresponding readings on, 363
Saletan, L. T., 844, 853, 856
Sallans, H. R., 242–244, 246, 639–641, 887
*Salmonella aetrycke,* 567
  *typhosa,* 797
Salting of cucumbers, 343, 344
Sandegren, E., 88
Sanders, G. P., 393, 397
Sandstedt, R. M., 498, 860
*Sarcina lutea* A.T.C.C. 9341, 794
Sarcina sickness, 166, 167
Sarett, B. L., 671
Sauerkraut, 332–340
  cabbage for, composition, 332
    salting, 333
    varieties, 332
  commercial manufacture, 332–336
  covering during fermentation, 333, 336
  definition, 332
  fermentation, 333–336
    function of salt, 335
    lactic acid bacteria associated with, 333–335
    optimum temperature, 336
    products, 333
    starters, 334, 335
  off flavors, 337
  references, 338, 339
  spoilage of, 336, 337
    dark, 336
    pink, 337
    rotted, 337
    slimy, 337
    soft, 337
Savard, K., 229
Savary, J., 498
Scalf, R. E., 109, 837, 858
Schade, A. L., 214, 215
Schaefer, B., 497
Schaefer, W. C., 372
Schardinger, F., 250, 295
Schattenfroh, A., 250
Schatz, A., 805, 806
*Schizophyllum commune,* 648
*Schizosaccharomyces octosporus,* 1
Scheele, K. W., 533, 637
Scheffer, M. A., 399, 401, 404
Scheme for butyl alcohol fermen
  276, 277
Scheunert, A., 85
Schmidt, F., 564
Schmidt, M., 662, 663
Schneider, R., 152
Schnellen, C., 510–512
Schoen, M., 250
Schoene, L., 844, 866, 867
Scholler, H., 875
Scholler-Tornesch process for wood
  charification, 875, 876
Schön, K., 460
Schopfer, W. H., 221, 698
Schreyer, R., 545, 546, 579
Schultz, A., 497
Schultz, A. S., 37
Schultz, J. S., 895
Schutte-Koerting jet heater, 838
Schwaiger, F. H., 171
*Schwanniomyces,* 35
  *occidentalis,* 14, 15
Schwarz, D., 240, 241
Schwarz, R., 152, 157
Schweiger, L. B., 546, 562
Schwimmer, S., 862
Sclerotia, 526
Sclerotiose, 524, 695
Scott, C. E., 814
Scott, D., 671
Screening techniques, 766
Screening test (fig.), 767, 768
Scum control, 352, 353
Seagram & Sons, Joseph E., 203, 838
Sedoheptulose, formula, 615
Seitz filter, 381, 383
Seneca, H., 800
Senior, J. K., 295, 297, 298
Senkus, M., 422, 423
Sensitivity tests for antibiotics, 794–796
  methods, agar-diffusion, 796
    agar-dilution, 795
    broth-serial-dilution, 795
Senti, F. R., 373
Sephton, H. H., 543
Serban, F., 229
Seromycin (*see* Cycloserine)
*Serratia indica,* 421
  *marcescens,* 400, 401, 420, 421, 483, 4
    715, 717, 805

*Serratia marcescens,* anaerobic dissimilation of glucose by, 421
  production of 2,3-butanediol by, 420, 421
Sery, T. W., 388
Seto, F., 619
Severson, G. M., 845–853, 855, 866
Sfat, M. R., 887, 894–896
Shah, S., 656
Shaker table (fig.), 775
Shankman, S., 301
Sharpe, E. S., 713, 715–718
Shaw, R. H., 474
Sherman, J. M., 351, 474–477
Sherrard, E. C., 872
Sherry, 175, 180, 181, 186
Shifrine, M., 39, 40
*Shigella gallinarum,* 805
Shimada, S., 620
Shimwell, J. L., 166, 167, 169, 428
Shiveler, G., 813
Shocho, 678
Shoyu, 678
Shriner, R. L., 867
Shu, P., 546, 551, 552, 562, 572, 666, 894
Shu method for citric acid, 551, 552
Shull, G. M., 723, 736, 738, 740, 741, 746–748, 750, 752, 788, 897
Shurter, R. A., 374–376, 894
iebel, R. V., 86
iebenäuger, H., 565
ilcox, H., 806
ilver, 324
ilverman, M., 399
imonart, P., 626
impf, K., 229–232, 240
impson, F. J., 672
ngh, J., 656
ngh, K., 73, 227
ngruen, E., 86
olander, J. R., 547
olander, N. O., 288, 289, 464
kinner, C. E., 37, 762
later, L. E., 442–445
loan, J. W., 371
medley-MacLean, I., 93, 94
miley, K. L., 221
ith, A. K., 676, 677
ith, C. E., 484
ith, C. G., 799
ith, E. L., 482
ith, G., 516, 518
ith, G. N., 797
ith, L. A., 121, 858
ith, L. T., 306, 323, 324
ith, N. R., 488
ith, W. L., Jr., 813
its, B. L., 610, 611
Smythe, C. V., 131, 673, 720
Snell, E. E., 301, 307
Snell, J. F., 73, 79, 80
Snell, N. S., 484, 488, 489, 491, 492
Snell, R. L., 546, 562
Snog-Kjaer, A., 301
Snyder, E. G., 671
Sobin, B. A., 800, 808
Sodium acetate, 313
Sodium bicarbonate, effect on *Cl. butylicum* fermentation, 290
Sodium carboxymethylcellulose, 358
Sodium fluoride, 130, 138, 142
Sodium gluconate, 590–594
  production, 590–593
    flow sheet (fig.), 590
    media used, 591
Sodium lactate, 324–326, 476
Sodium lauryl sulfate, 591
Sodium lauryl sulfonate, 621, 774
Sodium pectate, 358
Sodium penicillins, structure (table), 803
Sodium pentachlorphenate, 862
Sodium pyruvate, 292
Sodium sorbate, 352, 356
Softening of cucumber salt stock, 357, 358
Sohns, V. E., 380, 382, 383, 385, 386
Solomons, I. A., 805
Soltero, F. V., 777
Solvent formation by *Cl. acetobutylicum,* 270
Somers, G. F., 673
Sorbic acid as yeast inhibitor, 351, 352, 356
Sorbitol, 455–459
Sorbose, 456–459
  formula, 456
  production, 456–459
    effect of sorbitol concentration on, 457
    on semi-plant scale, 458, 459
    by submerged growth, 457–459
    by surface growth, 456–457
Sorgho, 170, 171
Sorghum, 503
Sour pickles, 341, 354, 355
Southcott, B. A., 810
Soy sauce, 673, 675–678, 681
  brine fermentation, 676, 677
    biochemical changes, 677, 678
  chemical, 677
  chemical composition and flavor, 678
  Chinese, 675–678
  preparation of kojies for, 676, 677
  references, 681
Soybean cake, 716
Soybean meal, 485, 486, 677, 859

- Soybean oil, 486, 508, 594
  - as antifoam agent, 508
- Soybean protein, 677
- Soybeans, 675–678
- Spangler, R. L., 338
- Spanish clay, 184, 437
- Speakman, H. B., 267, 273, 298
- Speakman inorganic salts, 477
- Spencer, J. F. T., 242–244, 246, 639–641
- Spencer, J. V., 810
- Sperber, E., 72
- Sphagnum moss, 543
- Spiculisporic acid, 525, 683, 690
  - formula, 690
- Spinulosin, 525, 684, 694
  - formula, 694
- Sporangium, 517
- Spore heads, 526
- Spontin (*see* Ristocetin)
- Sporulation media, 584, 591, 599, 773, 774
- Sporulation research, 22
- Squibb, E. R., and Sons, 801
- Sreenivasaya, M., 229
- Srinivasa, R. S., 770
- Stacey, M., 388
- Stadelman, W. J., 810
- Stahly, G. L., 399, 416
- Stanier, R. Y., 399, 401, 404–406, 408, 409, 412, 416–418, 673
- Stanley, A. R., 723
- Stansly, P. G., 804
- *Staphylococcus aureus*, 483, 766, 769, 804, 805, 809
  - A.T.C.C. 6538P, 789, 794, 795
- Stapley, E. O., 229–232, 240, 728
- Starch, nature of, 836
- Starch-saccharifying activity, 502
- Stark, W. H., 109, 114–116, 121, 837, 858–860
- Starkey, R. L., 91, 814
- Stauffer, J. F., 770, 771, 773
- Steam-sealed sampling system for fermentor (fig.), 892
- Stearns, T. W., 650, 652, 653
- Steel, R., 546, 553, 554, 556–563, 569, 570, 887
- Stefaniak, J. J., 770, 781, 807
- Steffen's molasses, 712
- Steinberg, M. P., 88–91
- Steinberg, R. A., 518
- Stelling-Dekker, N. M., 37
- Stephens, R. L., 650, 652, 653
- Stephenson, M., 160
- Sterigmata, 523
- Steroids, 723–761
  - basic structure of $\Delta^4$-3-keto, 723
  - ergosterol, 228–232
  - hydroxylation of semicontinuously added progesterone (fig.), 729
- Steroids, microbiological transfo 723–761
    - by cleavage of side chain, 75
    - by dehydrogenation reactio 750
    - effects of variables on, 728, 7
    - by epoxidation reactions, 750
    - examples, 727–729
    - history, 723, 724
    - by hydrogenation reactions,
      - reduction, of $\Delta^4$ double bon
        - of $\Delta^{16}$ double bond, 747
        - of side-chain aldehyde gro
        - of side-chain ketone, 747
    - by hydroxylation reactions,
      - 1α-, 730, 731
      - 6β-, 731, 732
        - examples (table), 732
      - 7-, 733, 734
        - examples of (table), 733
      - 8- (or 9-), 734, 735
      - 10-, 735
      - 11α- (table), 736, 737
      - 11β- (table), 738
      - 14α-, 739–741
        - examples (table), 740, 741
      - 15-, 741, 742
      - 16α-, 742
      - 17α-, 742–744
        - examples (table), 744
      - 21-, 745, 746
        - examples (table), 745, 746
    - methods, 724–729
    - some media used (table), 727
  - names, some synonyms for, 724
  - preparation of some cortical and adal hormones, 751, 752
  - references, 758–761
  - some microbes capable of transfo (table), 753–757
  - some produced, from progest (fig.), 725
    - from Reichstein's compound S 726
  - types of transformations, 729–750
    - examples (table), 730
- Sterols of molds, 524
- Stetter, H., 673
- Stewart, E. D., 240, 241, 844, 853, 856
- Stiles, H. R., 252, 272, 307
- Stillage, 252, 256
- Stimpson, E. G., 236
- Stipitatic acid, 525, 684, 690
- Stodola, F. H., 504, 619–621, 643, 715–718
- Stokes, J. L., 430, 431
- Stokstad, E. L. R., 484
- Stone, D., 735
- Stone, R. W., 474, 478, 777, 778

Stoudt, T. H., 229, 240
Stout, H. A., 800
Strandskov, F. B., 804, 818
Strange and Graham, Ltd., 250
Straw, 663
*Streptobacterium dextranicum* NRRL B-1254, 387
*Streptococcus bovis*, 484
  *damnosus*, 167, 169
  *faecalis*, 226, 348, 349, 483
    A.T.C.C. 10541, 794
  *lactis*, 265, 302, 304, 306, 320, 334, 395, 475, 812
Streptolydigin, 763
*Streptomyces albidoflavus*, 482
  *antibioticus*, 482, 800, 816
  *aureofaciens*, 482, 484, 735, 757, 807
  *cacaoi*, 816
  *celluloflavus*, 817
  *colombiensis*, 482
  *erythreus*, 798
  *fradiae*, 724, 732, 738, 757, 799
  *griseus*, 482, 484
  *lavendulae*, 751, 752, 757
  *niveus*, 799
  *noursei*, 800
  *olivaceus*, 482, 484–487
    NRRL B-1125, 485, 487
  *omiyaensis*, 797
  *orchidaceus*, 797
  *orientalis*, 809
  *reticuli*, 817
  *rimosus*, 808
  *roseochromogenus*, 482, 742
  *roseoflavus*, 817
  *spheroides*, 799
  *venezuelae*, 797
treptomycin, 805, 806, 812, 815
  assay methods, 794
  formula, 805
  historical, 805
  production, 806
  properties, 805, 806
  references, 827–831
  types of organisms against which effective, 805
treptonivicin (*see* Novobiocin)
rohmaier, A. J., 421
rong, F. M., 307, 655
rontium lactate, 326
ubbs, J. J., 85, 457–459, 461–463, 506, 508, 579, 581, 583, 588–590, 593, 815
umpf, K., 673
btilin, 763, 813
ccinic acid, 133, 143, 416–418, 420, 421, 477–479, 510, 511, 525, 565–567, 569, 606, 690
  ormula, 690
*ns*-Succinic epoxide hydrolase, 643
Succinoxidase system, 568
Suchar CSP, 548
Sugg, J. Y., 379
Sugihara, T. F., 647–649, 650, 652
Sugisake, Z., 713–716
Sulfite liquor, 69, 70, 117, 257, 320, 321
  composition, 117
  ethanol production from, 117–119
  lactic acid production from, 320, 321
  treatment, 117
Sulfonated castor oil, 73
Sulfurous acid for hydrolyzing starches, 867
Sullivan, M. X., 654
Sumiki, Y., 619, 620, 622
Sumner, J. B., 673
Sweet pickles, 341, 354, 355, 360
Sweet potatoes, dehydrated, for ethanol production, 114, 115
Swift, G., 387
Swim, H. E., 236
Swiss cheese, 397
Switzer, R. G., 341, 355
Sylvester, J. C., 798, 806, 887
Synnematin B, 806
  references, 831
Synonyms for some steroids (table), 724
Szpilfogel, S. A., 746, 748
Szücs, J., 546, 550, 551, 554, 557
Szücs method for citric acid production, 550, 551
Szüecs, J., 647
  (*See also* Szücs)

Tabachnick, J., 239, 241, 242
Tabenkin, B., 462, 463, 504–508, 631, 634, 635
Taette, 170
Takahashi, J., 894, 895
Takahashi, T., 462, 626, 630
Tamari, 678
Tamiya, H., 518, 609, 614
Tannase, 637, 638
Tanner, F. W., Jr., 219, 220, 222, 224, 225
Tannin, 158, 159, 637, 638
Tanret, C., 228
Tantalum, 324
Tappi, G., 229
D-Tartaric acid, 465, 466
  formula, 465
  production, 465, 466
*meso*-Tartaric acid, formula, 642
Tarr, H. L. A., 484, 810, 811
Tatum, E. L., 264, 305, 308, 320, 474–476
Tauber, H., 673
Tax gallon, 102
Taylor, G. G., 314, 315, 317

Taylor, T. H. M., 770, 781, 785
Tejesen, S. G., 896
Tempel, A. E., Jr., 897
Tendler, M. D., 483
Terramycin (*see* Oxytetracycline)
Terrestric acid, 525, 684, 690
  formulas, 690
Terroine, E. F., 654
Terszakowec, J., 240
Testololactone, 725, 726
Testosterone, 742, 752
Tetracycline, formula, 808
Tetracyclines, 807, 808
  references, 832
Tetraene, 800
Tetragalacturonic acid, 239
Tetronic acid derivatives, 684
Tetsuia, K., 109
Thaysen, A. C., 85, 272
Thelophoric acid, 525, 691
  formula, 691
Thermometers, brewery, 170
Thiamin, 27, 226, 227
Thiamin chloride, 548
Thiamin hydrochloride, 45, 49
  formula, 49
Thies, W., 145, 579, 626
Thimann, K. V., 279, 479, 721
Thin stillage, 499, 500, 858, 859
Thiolutin, 763, 817
Thirty-liter-fermentor assembly (fig.), 782
Thom, C., 519, 521, 523, 526, 533, 697, 801
Thoma, W., 742
Thorn, J. A., 639, 894
Thorne, R. S. W., 44, 50
Tilden, E. B., 461, 498
Time-temperature chart of brewhouse operations, 152
Tirage, 186
Tittsler, R. P., 301, 397
Tiukow, D., 565, 566
Tobie, W. C., 198
Tochikura, T., 714, 716
Tolman, L. M., 202, 204
Toluene, 711
Toluquinone derivatives, 684
Tomiyama, T., 810
Top fermentation, 161, 170
Toravill, A., 813
*Torula cremoris,* 720
Torularhodin, 28
*Torulaspora rosei,* 349, 350
Torulin, 28
*Torulopsis,* 34
  *caroliniana,* 349, 350
  *holmii,* 349–351
  *lactis-condensi,* 16, 17
  *Torulopsis sphaerica,* 226
Tošić, J., 428
Toulmin, H. A., 389
Toulmin, H. A., Jr., 389
Tourne disease, 191
Tower-type fermentor, 554
  drawing of, 556
Traetta-Mosca, F., 609
Trafton, H. M., 800
Transacetylase, 568
Transaminase, 237
Transamination reaction, 721
Transfer rooms (fig.), 774
Traufler, D. H., 220
Trehalose, 26
Tributyl citrate, 781
*Trichoderma viride,* 696, 743, 744,
*Tricholoma nudum,* 648
Trichosporoideae, 34
*Trichosporon fermentans,* 16, 17
  *pullulans,* 71, 88, 91–93, 100
    fat production by, 91–93
*Trichothecium roseum* (*see* *Cep*
    *thecium roseum*)
Trigalacturonic acid, 238, 239
*Trigonopsis,* 34
  *variabilis,* 16, 17, 232
3$\beta$,7$\beta$,21-Trihydroxyallopregnane-20
    733
2,4,5-Trihydroxyphenylglyoxylic ac
    525, 691
  formula, 691
6$\beta$,17$\alpha$,21-Trihydroxy-4-pregnene-3,2
    dione, 726, 731, 732
11$\alpha$,17$\alpha$,21-Trihydroxypregnane-3,20
    dione, 726, 731
11$\alpha$,14$\alpha$,17$\alpha$-Trihydroxyprogesterone
    738, 740
Tritisporin, 525, 684, 693
Triton B-1956, 815
Trofimova, E. I., 567
Tropolone derivatives, 684
Troutman, M. C., 47
Tryptophan, 43, 44
  formula, 43
Tryptophol, formula, 44
Tsilensis, E., 83
Tsuchiya, H. M., 379, 381, 383–385
    484, 762, 858
Tsunoda, T., 714, 716
Tubes for continuous cooking (fig.)
Tucker, I. W., 844
Tulloch, W. J., 770
Turfitt, G. E., 723, 751
Turner, H. D., 872
Turner, W. B., 691
Tutwiler, T. S., 607
Tween 20, 622, 815
Twenty-liter fermentor (fig.), 888

Two-bu.-per-day continuous alcohol unit, 120–122
  flow diagram, 121
Types of steroid reactions brought about by microbes (table), 730
Tyree, J. T., 389
Tyrosine, 42, 44
  formula, 42
Tyrosol, 44, 142
  formula, 44
Tyrothricin, 762
Tytell, A. A., 695

Ugolini, F., 887, 897
Umberger, E. J., 588–590
Underkofler, L. A., 213, 260, 263, 267, 399, 404, 422, 454, 459, 460, 464, 465, 666, 845–853, 855, 858, 860, 862, 866, 867
Unger, E. D., 837, 838, 892
U.S. Department, of Agriculture, 109–112, 308, 323, 379, 382, 393, 457, 485, 533, 579, 598, 631, 642, 666, 675, 801, 845, 877, 879, 880, 888
  of the Treasury, 123, 148, 150, 154, 155, 171, 176, 195, 196, 200, 205
U.S. Forest Products Laboratory, 872, 874, 879–881
University of Tokyo, 620
Upham, S. D., 811
Upjohn Company, 775, 776, 784, 787
Urea, 224, 402, 490, 506–509, 548, 549, 558, 640, 649, 659, 661, 662, 718, 719
*Uromyces phaseoli typica,* 815
Ustic acid, 525, 691
  formula, 691
Ustilagic acid, 639–641
  formula (tentative), 639
  production, 640
    medium for, 64
  properties, 639
  references, 641
  use for synthetic musks, 640
*stilago maydis,* 710
  *zeae,* 639, 640
    medium for maintenance, 640
*stulina vulgaris,* 534, 687

laer, P., 197, 199, 204, 205
lerie James, J., 546
line, formula, 44
nadium pentoxide, 466
n Cleve, J. W., 372
ncocin (*see* Vancomycin)
ncomycin, 763, 809
  ferences, 832
ndeputte, J., 796
n der Lek, J. B., 131, 271–278, 285
Van der Walt, J. P., 28
Van der Westhuizen, G. C. A., 543, 598, 601, 605
Vanghelovici, M., 229
Van Lanen, J. M., 114, 222, 224, 225–227, 260, 666, 858, 859
Van Niel, C. B., 474–478
Van Tieghem, P. E. L., 637
Van Winkle, W., 801
Varianose, 524, 695
Variation in strains, 4
Vaughn, R. E., 351, 357, 428–431
Vegifat Y, 73, 490
Veldhuis, M. K., 348
Vercellone, A., 723, 746
Verhulst, J. H., 297
Vertical incubation cell (fig.), 850
Vertical mash mixer, 156
Vesicle, 523
Vienna process, 60
Viljoen, J. A., 332
Villemin, P. F., 762
Vincenty, C., 73, 598, 601, 602, 605
Vinegar, 432–453
  acidification, object of, 434
  aging, 437
  bottling, 437
  causes of spoilage in factory, 449–451
    darkening, 450
    eels, 450
    mites, 450, 451
  clarification of, 437
  composition of, 432
  composition of containers and equipment, 437, 438
  concentration of ethanol used, 434
  darkening, 450
  defects, 449–451
  definitions and standards, 432
    apple, 432
    cider, 432
    malt, 432
    spirit, distilled, 432
    sugar, 432
    wine, 432
  general requirements for making, 433
  generators with automatic control, 440–442, 448, 449
  German process, 439
  grains strength, 452
  history, 432, 433
  mechanism of acetic acid formation, 452, 453
  methods of manufacture, 438–448
    French, 438
    Frings', 439–441
    modern plant, 442–445
    processes, German, 439
      Mackin, 441, 442

Vinegar, methods of manufacture, processes, Orleans, 438, 439
    - quick, 439
    - submerged, 445–449
  - revolving generators, 442
- nature of raw materials used, 433
- nomenclature, 433
- oxygen in manufacture, 434, 435
- pasteurization, 437
- raw materials used, 433
- references, 469–473
- storage, 337
- supporting medium used, 435, 436
- temperature relations, 436, 437
- uses, 452
- yields, 451
  - calculation, 451, 452

Vinegar eels, 450
Vinegar flies, 451
Viomycin, 763, 794
Virtanen, A. I., 474, 478
Vischer, E., 742, 748, 749, 751
Visor, F. C., 812
Visser, J., 896
Visser't Hooft, F., 428, 431, 459
Vitamin $B_{12}$, 482–496
- assay, 492, 493
- formula, 483
- introduction to, 482
- microorganisms producing, 482–484
- production, 484–494
  - by *Bacillus megatherium*, 488–494
    - conditions for propagation, 490, 491
    - effect of temperature on growth and cobalamin synthesis (fig.), 492
    - isolation of cobalamin-producing strains, 489
    - media and nutrients, 490
    - recovery, 492
    - yields, 492
  - by *Streptomyces olivaceus*, 484–488
    - aeration and agitation, 487
    - flow diagram, 486
    - inoculation and fermentation media, 485
    - inoculum preparation, 485
    - recovery, 487, 488
    - sterilization of media and facilities, 486, 487
    - temperature of fermentation, 487
- references, 494–496
- uses, 494

Vitamin contents of some food yeasts, 27
Vitamin $D_2$, 232
Vitamin production by food yeasts, factors affecting, 226–228
Vitamins, 45, 48, 476
- structural formulas of som

Vogel, E. H., Jr., 171
Vogelbusch fermentors, 79
Vojnovich, C., 220, 222, 252, 601–605, 887
Volatile acids formed by *Cl. butylicum*, 269
Von Loesecke, H. W., 81, 534
Von Soden, O., 85

*WADC Technical Report* 52–3
Wadsworth, C. K., 355
Wagner, K. H., 85
Wakisaka, Y., 713, 714
Waksman, S. A., 484, 521, 546 562, 625–627, 635, 770, 762 799, 805, 806
Waldhof aeration wheel, 76, 7
Waldmann, R., 498
Waldmann process for amylases
Walker, R. D., Jr., 73, 79
Walker, T. K., 301, 428, 466, 468, 656, 658, 659, 695
Waller, C. J., 553
Wallerstein, L., 164, 497, 498
Wallerstein procedure for amyl
Wallick, H., 799
Walpole, G. S., 399, 401
Walsh, J. F., 319
Walter, F. G., 70
Walter, H. E., 397
Walton, R. B., 800
Wang, T. H., 678
Ward, G. E., 400–403, 484, 485, 508, 529, 600, 601, 605, 619, 635, 654, 658–660
Ward, L., 301, 348
Wassele, L. A., 805
Waste protein water, 83
Water-white lactic acid, 312
Waters, W. R., 546, 553, 554, 887
Watson, P. R., 388
Watson, R. W., 673
Webb, N. S., 30
Weber, J. F., 86
Weber, U., 239
Wegrich, O. G., 894
Wehmer, C., 533, 534, 538, 545, 626
Weinhouse, S., 568, 572
Weiser, H. H., 811, 813
Weizmann, C., 250, 263
Welch, H., 502, 797, 799, 802
Wells, P. A., 457–459, 461–463, 587, 593, 631, 634, 635
Welsch, M., 723
Wenck, P. R., 229, 232, 661

Wendland, R. T., 260
Werkman, C. H., 277, 285, 286, 290–292, 306, 327, 399, 416, 459, 474, 475, 478, 770
Wester, R. E., 815
Wetter, L. R., 671, 672
Wettstein, A., 723, 732, 735, 740–742, 751
Weyer, E. R., 263
Whales, preservation by antibiotics, 811
Wheat for ethyl alcohol production, 114, 115
Wheat bran, 499
Wheat grits, 307
Whey, 236, 394, 395, 475, 662
  for ethyl alcohol production, 109–112
Whiskey, 200–207
  composition, 201, 202
  Federal control, 205
  production, 201–205
    aging, 204, 205
      changes during, 204, 205
      quick, 204
    distillation, 204
    fermentation, 201, 202, 204
    flow diagram, 203
    raw materials, 201
  references, 206, 207
  standards of identity, 205
  types, 201
Whistler, R. L., 465
Whitaker, D. R., 672
White, A. G. C., 770
White, J., 61, 65–67, 70
White, M. G., 695
hiteside-Carlson, V., 388
hittier, E. O., 109–112, 474, 476, 477
ickerham, L. J., 33, 36, 38, 45, 49, 219, 354
ickerham special medium, 45
ickerham yeast morphology agar, 49
ieland, H., 453
iesen, C. F., 484
ikén, T., 287, 292
iley, A. J., 27, 73, 75–77, 86
ilham, C. A., 370
ilker, B. L., 770, 898
illaman, J. J., 695
lliams, A. E., 590
lliams, O. B., 813
lliams, R. T., 482
lliamson, S., 240
lkie, H. F., 109, 838
ison, F. A., 85
lson, P. W., 572
on, R. E., 893
daus acid, 723
disch, F., 452
dlan, H., 810

Wine, 174–194
  acetic acid in, 190
  acetic acid bacteria, 191
  aeration, 182
  aging, 183, 184, 186, 188
  appetizer, 175
  bouquet, 188
  California, analysis, 189
  "cap," 182
  chemical composition, 188–190
    fixed acids, 189
    volatile acids, 189
  citric acid, 190
  clarification, 184, 185
  classes and types, 174, 175
  cream of tartar in, 183
  defects, 191–193
    bitter, 192
    ferric casse, 192
    *L. hilgardii,* 192
    mannitol-forming bacteria, 192
    oxidase casse, 193
    slime-forming bacteria, 192
    Tourne, 191
    white casse, 192
  deficiencies, correction, 186
  definition, 174
  dessert, 175
  dry, definition, 174
  esters, 190
  fermentation temperature, 183, 185, 186
  fining of, 184
    bentonite, 184
    casein, 184
    egg white, 184
    gelatin, 184
    isinglass, 184
    Spanish clay, 184
    tannin, 184
  flow diagram of making, 180, 181
  flowers of, 184
  formic acid in, 190
  "free-run," 183
  gallon, 102
  grapes used for making, 177–179
    crushing, 177
  imports, 176
  lees, 183
  making areas, 174
  malic acid in, 190
  pasteurization, 184, 186
  phosphoric acid in, 190
  port, flow diagram of making, 180, 181
  potassium metabisulfite in, 180
  propionic acid in, 190
  racking, 183
  red table, making, 177–185
  references, 193–194

Wine, regions of production, 174
  sherry, 186
    flow diagram of making, 180–181
  sparkling, 185, 186
    definition, 174
    imports, 175
    production, 176
  still, definition, 174
    imports, 175
    production, 176
  succinic acid in, 190
  sugar concentration of must in, 177
  sulfur dioxide, 177, 180, 181, 190
  tannic acid in, 190
  tartaric acid in, 190
  white table, 175, 180, 181
  yeast starter, 182
Wine vinegar, 432
Winge, Ö., 31, 32
Winkler, A. J., 190
Winogradsky, S., 250
Winoker, J., 222
Winston, A. L., 332
Winston, K. B., 332
Winter, H. F., 814
Winthrop Chemical Co., 801
Wise, W. S., 895
Wiseman, R. F., 301
Wisseman, C., 797
Wisthoff, R. T., 861, 862
Witham, C. A., 388
Wittwer, S. H., 622, 623
Wohlgemuth, J., 502
*Wojnowicia graminis,* 745, 757
Wolff, I. A., 371, 373, 387, 388
Wood, H. G., 277, 307, 474, 477, 478
Wood, T. R., 484
Wood, W. A., 279, 328
Wood hydrolyzates, 288, 289, 403
Wood saccharification, 871–882
  historical, 872, 873
  processes for, Bergius-Rheinau, 874, 875
    Giordani-Leone, 877
    Madison wood-sugar, 877–881
    modified Rheinau, 881, 882
    Scholler-Tornesch, 875–877
  references, 882–884
Wood sugar, 260
  for ethyl alcohol production, 118, 120
  stillage, 80, 81
Wood-Werkman reaction, 572
Woodman, A. G., 201
Woodruff, H. B., 770, 802
Woodward, C. F., 324, 325
Woodward, J. C., 546
Woodward, T. E., 797
Work, E., 710, 711
Wrenshall, C. L., 813
Wright, E. T., 800
Wright, W. W., 799
Wustenfeld, H., 563
Wynne, A. M., 265

*Xanthomonas campestris,* 505
  *juglandis,* 814
  *stewartii,* 505
  *vesicatoria,* 814
Xanthone derivatives, 684
D-Xylonic acid, 508, 509
Xylose, 264, 296, 416–418, 610,
  fermentation, by *Aeromonas*
      *drophila,* 418
    by *B. polymyxa,* 416

Yabuta, T., 609, 613, 620, 622
Yakeishi, N., 715
Yamada, K., 894, 895
Yamada, K. J., 70
Yamada, M., 239
Yamamoto, A., 895
Yamasaki, I., 503, 504
Yazaki, H., 716
Yeast extract, 275, 286, 287, 290, 2
    382, 406, 409, 412, 414, 417, 4
    430, 454, 458, 464, 465, 475–4
    490, 491, 640, 716, 719
Yeast extract–malt extract mediu
Yeasts, 10–59
  amino acid composition, 26
  ascospores, 21
  ashes, composition, 28, 46, 47
    elements in, 47
  budding, 10, 11, 62
  cell wall, 18
  chemical composition, 25–28
  chromosomes in, 22
    diploid number, 23
    haploid number, 23
  classification, 33–37
  contents of cell in, 18, 19
  enzymes in, 236–240
  esters, 239, 241, 242
  fat production by, 87–95
  flocculation, 49–51
  genetics, 22–25
    references, 57
  identification, 32, 33
  isolation of pure cultures, 28–31
    media, 30, 31
    methods, dilution-plate, 30
      Hansen, 29
      Lindner, 30
      micromanipulator, 30
  media, 30, 31, 45, 49
  nitrogen assimilation by, effect
      vitamins on, 44, 45

Yeasts, nitrogen-containing compounds in, 25
  nucleus, 22
  nutrition, 40–49
    carbon, 40, 41
    mineral requirements, 45–47
    nitrogen, 41–45
    vitamins, 48, 49
  occurrence and distribution, 9, 12, 13
  products formed by, 52
  propagation, 60–101
  references, 52–59
  reproduction, 10, 11
  shapes of cells, 14, 16
  sizes, 14, 16
  spores, 19
    isolation, 31, 32
  sporulation, 19–21
    basic research on, 22
    inhibition, 20
    methods of inducing, 21, 22
    optimum temperatures, 20
  utilization of amino acids by, 42, 43
  vitamin production by, 218–228
Yellow oil, 267
Young, H., 238
Young, H. C., 814
Young, W. J., 128, 129

Zaffaroni, A., 728, 745, 746
Zarow, A. I., 115, 116
Zaumeyer, W. J., 814, 815
Zeo-Karb H, 561
Ziegler, D. W., 809
Ziegler, F., 810
Zinc lactate, 320, 323
Zobrist, F., 292
Zook, H. D., 229
Zygospores, 517

TECHNOLOGICAL RESEARCH INSTITUTE
LIBRARY
MYSORE

10/8/89

C.F.T.R.I. LIBRARY, MYSORE – 13

4/97

**CFTRI LIBRARY, MYSORE - 13**

**Acc. No.** 5906

**Call No.** GV-9x

**Please return this publication on or before the last due date stamped below incurring overdue charges.**

**To be issued from:**

| DUE DATE | RETURNED ON | DUE DATE | RETURN |
|---|---|---|---|
| 5/1/98 | 5/1/98 | 4-5-2K | 24/4/2 |
| 5/5/98 | 21.4.98 | 10.9.2K | 28/8 |
| 6/3/98 | 10/3 | 26/6/01 | 13/6/0 |
| 9.9.98 | 26/8 | 31.08.01 | 28/ |
| 9.9.98 | 9.9.98 | 6.9.01 | 13/9 |
| 3/12/98 | 3.12.98 | 4/9/02 | [illegible] |
| 29/12/98 | 21/12/98 | 7.6.2002 | [illegible] |
| 9/1/98 | 14/1/99 | 15/8/02 | 1/8/02 |
| 20/2/99 | 7.2.99 | 30/8/02 | 2/9/2 |
| 27.2.99 | 28/2/99 | 16/9/02 | 2/9/2 |
| 30/3/99 | 7/7/99 | 7.11.02 | 31.10.0 |
| 6.9.99 | 27/8/99 | 13.9.03 | 11.9.03 |
| 31/7/01 | 21.7.2001 | 19.8.2004 | 10.8.04 |

Acc. No. 5906
Call No GV-9X
Prescott (S.C) IV 59
ustrial

C.F.T.R.I.